REMARKS

In an office action mailed February 5, 2010, claims 26-41 have been rejected. In response, Applicants have amended claims 26, 33 and 40, cancelled claims 30, 31 and 41, and provide the herein remarks. Claims 26-29, and 32-40 are pending examination. Entry of the herein amendments and remarks is respectfully requested.

Statement of the Substance of the Interview

Applicants would like to extend their gratitude to Examiners Long and Woitach for their willingness to participate in an in-person interview with inventor, Victor van Beusechem, European Attorney, Mark Einerhand, and Lauren T. Emr, on May 19, 2010 at the USPTO.

During the interview, Examiners Long and Woitach were gracious enough to allow Dr. van Beusechem to present a series of slides that compared adenoviruses of the cited art with the claimed adenovirus, and to discuss publications that support "a poor likelihood of success" when combining the teachings of the cited art.

The language of claim 26, and the cited documents by Fueyo, Lin and Hallenbeck were discussed. Although an agreement was not reached during the interview, Applicants appreciate the guidance and feedback provided by the Examiners and are hopeful that the herein response will convince the Examiners of the patentability of the claims.

Claim amendments

Claim 26 has been amended to change the article preceding "recombinant adenovirus" from "a" to "the." The relevant part of claim 26 now reads:

> "…whereby said restoring factor induces accelerated cell lysis and/or a faster release of virus progeny when compared to ~~a~~ the recombinant adenovirus lacking said coding sequence,…"

This amendment was made to clarify that the comparison is made with the recombinant adenovirus lacking said coding sequence.

Claim 33 has been amended to correct a spelling error. Claim 40 has been amended to correctly refer to a sequence. No new matter has been added.

Sequence Compliance

The office action points out that claim 40 allegedly does not conform to the sequence rules. In response, Applicants have amended claim 40 to conform to the sequence rules by reciting "SEQ ID NO: 5."

Rejections Under §103

Claims 26-27 and 30-41 have been rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Fueyo et al. in view of Lin et al. Claims 26-33, 35-39 and 41 have been rejected under §103 (a) as allegedly being unpatentable over Hallenbeck et al., in view of Lin et al. Applicants respectfully disagree with the rejections.

According to the Examiner, Fueyo disclose a replication competent adenovirus having the adenovirus E1B-19kDa and E1B-55kDa proteins. The Examiner notes that "it is clear from Fueyo that their adenovirus induces apoptosis in p53 deficient cells…"

According to the Examiner, Hallenbeck disclose a tumor specific replication restricted adenovirus having "the essential E1A gene…expressed form a tumor-specific promoter." The Examiner recognizes that neither Fueyo nor Hallenbeck disclose an adenovirus having at least one mammalian restoring factor functional in restoring the p53 apoptosis pathway.

The Examiner cites Lin as allegedly disclosing an adenovirus p53 variant that purports to restore function in cells that lack endogenous p53 and induce apoptosis. Therefore, the Examiner asserts that Lin teach an adenovirus that contains a mammalian restoring factor.

The Examiner alleges that it would have been obvious to a person of ordinary skill in the art to incorporate the tissue specific replication conditional control features of Fueyo or Hallenbeck into the adenovirus p53 construct of Lin, and arrive at the claimed invention. For the following reasons, Applicants strongly disagree.

Lin is concerned with a replication defective adenovirus that contains a mutant p53 gene. The mutant p53 is deficient in MDM2 binding. The mutant p53 of Lin is basically the same as a wild type p53 ("wt p53) with the exception that it does not efficiently bind MDM2 (Lin et al, page 5896, right hand column section "The transcriptional activation activity of p53 14/19"). Lin

states that the adenovirus with the mutant p53 can induce the endogenous p53 regulated gene products, which are involved in growth arrest and apoptosis at similar levels to adenovirus with wt p53 (last sentence of the mentioned section on page 5896).

The way that replication defective p53 containing adenoviruses work is detailed, among others, in a review by McCormick (2001), a copy is attached hereto as Exhibit A. Figure 2 of McCormick depicts a viral vector containing a tumor suppressor gene. The effect of the virus in a normal cell is presented and the effect of the virus in a tumor cell is presented. In normal cells, there is no effect whereas the tumor cell is triggered into cell cycle arrest or apoptosis.

The concept of oncolytic viruses is very different. McCormick explains in figure 4 the mechanism of action of conditionally replicating viruses. These viruses are tailored to replicate specifically in tumor cells and not in normal cells. The idea behind the use of replication competent viruses is that the virus spreads in the tumor to neighboring cells that were not infected in the original hit. In this way, the effect of the treatment is greater.

McCormick also gives a detailed description of the actions of oncolytic viruses on the pRB and p53 pathways in normal cells and in tumor cells (page 135 paragraph bridging left and right hand columns and Fig 5 on page 137). A replication competent adenovirus needs to modify the pRB pathway in a cell in order to activate adenovirus E2 gene expression and host cell S-phase. The activation of the S-phase allows replication of the adenovirus, presumably because it

provides host factors needed for DNA synthesis. On the other hand, replication competent adenoviruses need to block p53 function to replicate efficiently.

Fig 5 shows the connection between RB, E2F, ARF, MDM2 and p53. In a normal resting cell, E2F is inactive because it is sequestered by RB. Adenovirus E1A binds to RB which as a result releases E2F. The released E2F then activates adenovirus E2 expression and S-phase. One of the targets of E2F is ARF.

E2F activates ARF which in turn inhibits MDM2. MDM2 targets p53 for destruction. A high level of MDM2 thus depletes the cell of p53. Inhibition of MDM2 on the other hand activates p53. Active p53 causes abortive replication, apoptosis and cell-cycle arrest. The activation of p53 via MDM2 and ARF can thus be seen as a sanity check of normal cells. Adenoviruses need to block the action of the p53 protein in order to replicate efficiently.

Adenoviruses achieve this elimination of p53 by producing the early region protein E1B 55k. With the help of adenovirus E4 orf6 this protein targets p53 for destruction. Adenoviruses that lack E1B-55k such as the ONYX-015 virus cannot destroy p53 and therefore cannot replicate in normal cells.

In tumor cells, p53 itself is defective or the pathway connecting pRB function to p53 function is broken. As p53 is not functional in these cells, adenoviruses do not need to shut down p53 function, because it is not there from the beginning. Thus adenoviruses that can't shut down

p53 function are replication deficient in normal cells, which have a functional p53 pathway, and are replication competent in tumor cells wherein p53 function is absent.

The Fueyo virus has an E1A protein that cannot bind pRB. In normal cells, it is unable to release E2F from RB and thus cannot activate its E2 gene and push a normal cell into S-phase (Fueyo et al, page 2, introduction). Tumor cells have a defective pRb pathway, in which pRb is either absent or mutant, or constantly in a hyperactive state. Consequently, they regularly undergo S-phase during cell cycle and do not need the help of adenovirus E1A to enter S-phase. The Fueyo virus is thus a conditionally replication competent virus that can replicate in tumor cells, but that cannot replicate in normal cells.

The Hallenbeck virus does in principle the same thing as FueyO. In the Hallenbeck virus, E1A is under a tumor specific promoter (abstract). The virus does not produce E1A in normal cells and thus is not able to push the normal cell into S-phase. On the other hand, E1A is produced in (hepatocellular carcinoma) tumor cells and in these cells the virus is replication competent.

Both the Fueyo virus and the Hallenbeck virus do not activate the pRB pathway in normal cells and thus do not activate p53 dependent apoptosis in these normal cells. In tumour cells, the viruses also do not activate p53 dependent apoptosis. This is impossible as p53 is not active in these cells, either as a result of p53 mutation or due to a disconnected pRb->p53 pathway.

How then do these viruses kill the tumor cells? Several mechanisms are suggested in the art. Adenovirus E4 orf4 is implicated in a p53 independent apoptosis pathway (Branton and Roopchand, 2001). A copy of the same is attached hereto as Exhibit B.

Hassan et al (2004) claim that the conditionally replicating adenoviruses described by Fueyo kill tumor cells via a basic apoptotic machinery-independent mechanism that resembles necrosis-like programmed cell death (abstract). A copy of Hassan et al. is attached hereto as Exhibit C.

Hong Jiang et al (2007) have investigated the cell death mechanism of the Fueyo virus and come to the conclusion that this virus kills tumor cells via adenovirus-mediated cell death via autophagy (abstract). A copy of Hong Jiang is attached hereto as Exhibit D.

It is thus clear that replication competent adenoviruses need to activate S-phase in a normal cell in order to replicate. By doing so, the adenoviruses activate p53 which in turn inhibits adenovirus replication. Replication competent adenoviruses overcome this p53 mediated block by targeting p53 for destruction (through the action of E1B 55K and E4 orf6).

Conditionally replicating adenoviruses do not activate the pRB->p53 pathway in normal cells and thus do not push these cells into S-phase. The conditionally replicating viruses are

replication competent in tumor cells that are already in S-phase and do not have an active p53 apoptosis pathway.

At The Time of Invention, No Motivation to Combine the Teachings Of Lin With Fueyo or Hallenebeck Existed

At the time of the invention, it would not have made sense or been obvious to the person skilled in the art to combine the teaching of Lin et al with the teaching of either Hallenbeck or Fueyo.

As mentioned above, replicating adenoviruses do not kill cells in a p53 dependent fashion. p53 is effectively inhibited and degraded by adenovirus-encoded proteins (E1B 55K, E1B19K, and E4orf6). Replication competent adenoviruses kill cells by other means.

Thus, there would not have been any motivation (simply because it would not have made sense) to express p53 in a replication competent adenovirus. Moreover, even if one would succeed in overcoming p53 inhibition by the adenovirus-encoded proteins, then p53 expression would inhibit virus replication and thereby negate the entire concept of a conditionally replicating competent adenovirus (see e.g. review by Hermiston and Kuhn, 2002: filed with our response of June 22, 2009). Another copy of Hermiston and Kuhn is attached hereto as Exhibit E.

At the Time of Invention, There Was No Reasonable Expectation of Success Upon Combining the Teachings of Fueyo or Hallenbeck with Lin.

In the section "Tumor suppressors and antioncogenes as therapeutic transgenes" on page 1026, Hermiston et al state that, while "numerous preclinical studies using replication-defective viruses have shown that restoration of tumor suppressor function [...] slows tumor growth and/or leads to apoptosis or cancer cell death. Not much is to be expected from conditionally replicating viruses carrying such genes, most importantly because this "is also likely to attenuate viral replication." Furthermore, expression of such genes "may compromise the engineered or endogenous tumor selectivity mechanism of the oncolytic virus or the viruses' ability to replicate in the target tumor cell. The former would be detrimental to safety, and the latter to efficacy."

Thus, at the time of the filing of the application, there was no reasonable expectation of success of conditionally replicating adenoviruses carrying tumor-suppressor genes or anti-oncogenes. The effect of the combination as established in the present invention could not be predicted. In fact, the art taught away from the present invention as the combination was thought to be ineffective.

With respect to the interview summary and the comments of the Examiner concerning the replication of the virus in Figure 2 of the instant application, it is pointed out that the virus expressing p53 in Figure 2 shows accelerated progeny virus release as compared to the GFP control virus in all tested cell lines, including SaOs-2.

For all cell lines, shortly after infection more viruses released into the culture medium were found in the presence of p53 than in the presence of GFP. In SaOs-2 cells, on day 3 the titer of the p53 virus is approximately 10x higher than the GFP control. The titer of the p53 virus remains higher than the control virus until day 6. The titer of the p53 virus levels off after day 6 whereas the GFP virus titer continues to increase after that.

Thus, the final titer achieved after infecting SaOs-2 cells is higher with the GFP virus. The SaOs-2 cell line is exceptional only in this aspect. On the other cell lines, the final titer is similar or higher with p53 than with GFP. However, in line with the observations on the other cell lines, the replication of the p53 virus is faster also on SaOs-2 cells. Figure 2 thus supports the notion that a virus according to the invention induces a faster release of virus progeny compared to a similar virus lacking a coding sequence for the restoring factor.

It is now believed that the application is in condition for allowance. If the Examiner believes a telephone discussion would be beneficial to resolve any outstanding issue, he is invited to contact the undersigned without hesitation.

Respectfully submitted,

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Exhibit A

CANCER GENE THERAPY: FRINGE OR CUTTING EDGE?

Frank McCormick

Direct targeting of cancer cells with gene therapy has the potential to treat cancer on the basis of its molecular characteristics. But although laboratory results have been extremely encouraging, many practical obstacles need to be overcome before gene therapy can fulfil its goals in the clinic. These issues are not trivial, but seem less formidable than the challenge of killing cancers selectively and rationally — a challenge that has been successfully addressed.

Gene therapy offers tremendous promise for the future of cancer treatment. This technology, more than any other, takes direct advantage of our new understanding of cancer at the molecular level and has been exploited to develop new strategies for killing cells selectively or arresting their growth. However, despite the promise of safe and rational treatment, many researchers have serious doubts as to whether this is a viable approach. Is this view accurate? Or does gene therapy have a future in mainstream clinical oncology?

The field of cancer gene therapy embraces a range of ideas and technologies — from direct attack on tumour cells to harnessing the immune response to tumour antigens (FIG. 1). Here, we will restrict our discussion to direct attack on tumour cells, which requires an understanding of the intracellular signalling pathways that have gone awry in tumour cells; the success of immunotherapy depends on understanding the complex interactions between tumour cells and the immune system — a distinct intellectual challenge that is beyond the scope of this review. There are three types of weapon in this direct offensive, and we will consider each of these in turn.

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Tumour suppressors and oncogenes

In the most direct application of cancer gene therapy, tumour-suppressor genes are expressed in cancer cells in which these genes are defective, resulting in cell death or growth arrest (TABLE 1; FIG. 2). This concept is based on two assumptions, neither of which should be taken for granted. The first is that restoration of a single genetic defect will be effective in inhibiting tumour cells that have many additional defects. Perhaps surprisingly, for each of the principal known tumour suppressors — *APC*[1], *RB*[2,3], *INK4A* (REFS 4,5), *PTEN*[6–8], *ARF*[10] and *TP53* (REFS 11,12), — it turns out to be true: expression of each of these genes in tumour cells *in vitro* causes an acute change in cell

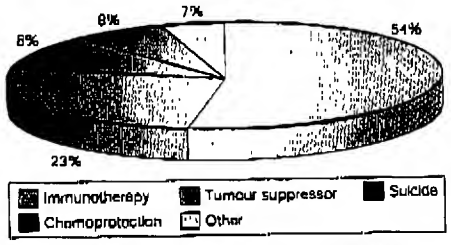

Figure 1 | **Cancer gene therapy and immunotherapy trials currently listed as open by the US Recombinant Advisory Committee.** Over half of all gene-therapy-based protocols in the United States (113 currently open) are aimed at boosting the immune response to tumour antigens. Trials in melanoma alone account for 54% of immunotherapy trials. Delivery of the tumour-suppressor gene *TP53* accounts for the next largest group, followed by suicide gene delivery, in which viral vectors deliver enzymes that activate prodrugs to toxic products that kill tumour cells and their neighbours. Most of these use herpes simplex virus thymidine kinase (HSV-tk), which activates the prodrug ganciclovir. Chemoprotection is an indirect approach in which bone marrow cells are infected with viruses that protect them from the toxic effects of chemotherapy, by expressing drug-resistance genes

Table 1 | **Effects of expressing tumour suppressors in tissue culture and in mouse models**

Gene product	Function	Expression in cell lines	Expression in mouse models	References
INK4A	Blocks cell cycle by inhibiting CDK4	Growth arrest (some evidence of resistance)	Tumour suppression	4,5,91
INK4A–KIP1 fusion	Blocks cell cycle by inhibiting CDK4 and CDK2	Apoptosis	Regression	92
RB	Blocks cell cycle by repressing E2F	Growth arrest	Tumour suppression	2,3,93
p130	Blocks cell cycle by repressing E2F	Growth arrest	Regression	94
ARF	Protects p53 by inhibiting MDM2	Growth arrest	Not done	10
p53	Promotes cell-cycle arrest and apoptosis	Growth arrest; increased radiosensitivity	Tumour suppression; reduced metastasis	95–98
PTEN	Degrades 3-phosphorylated phosphoinositides, which activate growth and survival pathways	Growth arrest; apoptosis; increased radiosensitivity	Tumour suppression or no effect	6–9
APC	Targets β-catenin for degradation	Apoptosis	Not done	1
BRCA1	Genome integrity	Growth arrest or apoptosis	Tumour suppression	13,14

APC, adenomatous polyposis coli; RB, retinoblastoma.

physiology and gene expression, resulting in cell-cycle arrest or death (TABLE 1). In addition to validating the concept of this form of gene therapy, these experiments clearly illustrate the selective advantage of losing tumour-suppressor gene expression in tumour development.

For genes that primarily affect genome integrity, such as *BRCA1* and *BRCA2*, this assumption is less likely to be valid. These genes have their selective effects during initiation of the disease, and would be expected to have little, if any, effect at later stages. But surprisingly, retroviral expression of *BRCA1* causes growth arrest or apoptosis in breast cancer and ovarian cancer cells, even though these cells already express the wild-type *BRCA1* gene[13,14]. The mechanism of these effects is not understood, and, unfortunately, has not borne fruit in the clinic: Phase I and II clinical trials of a *BRCA1*-expressing vector in women with ovarian cancer yielded no clinical responses, although this might have been because the vector was not stably expressed[15].

Some tumour-suppressor genes affect genome stability as well as cancer-cell survival and growth. Loss of *TP53* increases the rates of spontaneous cancers tremendously[10]. Likewise, *APC*, which is mutated in most colorectal cancers, might be involved in maintaining genome stability[17,18]. Does this have any bearing on their effects when expressed in tumour cells? Expression of either *TP53* or *APC* in tumour cells causes rapid growth arrest or apoptosis. Their potentially therapeutic value seems to depend on these acute effects rather than on their status as guardians of genome integrity.

A second assumption about the mechanism of direct attack by tumour suppressors is that collateral delivery of these genes to normal tissue will have little effect because these genes are expressed in normal cells anyway and are appropriately regulated in those cells. This issue has not been addressed rigorously in model systems. One reason for this is that it is difficult to predict which normal tissue would be exposed to the transgene. The normal counterpart of the tumour itself would seem a logical choice for experimental investigation, but the toxic effects of the gene are more likely to be seen in cells that are exposed to the highest levels of the vector, such as the liver or vascular endothelial cells. Nevertheless, delivery of *TP53* to normal bronchial epithelial cells showed no effects on cell growth, with a 2–3 log THERAPEUTIC WINDOW relative to tumour cells[10].

These results go some way towards validating the notion that delivery of a tumour-suppressor gene could have therapeutic value, but the effects were expected to be cell autonomous, with little hope of any effect on surrounding, uninfected tumour cells. This meant that every cell in a tumour would need to be infected — an enormous technical hurdle, particularly for disseminated cancers. But surprisingly, such an effect (defined as a 'bystander effect') might well account for some of the efficacy of an adenovirus that expresses *TP53* in solid tumours. One interesting explanation is that p53 is anti-angiogenic: it downregulates expression of vascular endothelial growth factor (VEGF)[20,21], and upregulates expression of thrombospondin — a potent inhibitor of angiogenesis[22]. Forced expression of *TP53* could therefore lead to toxic effects on uninfected neighbours by starving them of oxygen and nutrients. p53 regulates other gene products that are capable of a bystander effect. One of these is insulin-like growth factor 1 binding protein (IGF1BP)[23], which neutralizes the anti-apoptotic effects

THERAPEUTIC WINDOW
The range of drug concentrations over which a drug has a therapeutic effect without having unacceptable toxicity.

RETROVIRAL VECTOR
Gene-therapy vector derived from a retrovirus. The *gag*, *pol* and *env* genes, necessary for replication of the virus, are replaced with a therapeutic gene, preventing viral replication.

ADENOVIRAL VECTOR
Gene-therapy vector derived from an adenovirus. Genes necessary for replication of the virus can be deleted to make replication-defective vectors.

ANTISENSE OLIGONUCLEOTIDE
An oligonucleotide that is complementary to a portion of an mRNA. It binds to the mRNA and arrests translation by physical blockade of ribosomal machinery and/or by activation of endogenous RNases.

RIBOZYMES
RNA molecules with catalytic activity. They can be engineered to cleave specific mRNAs, thereby blocking gene expression at the mRNA level.

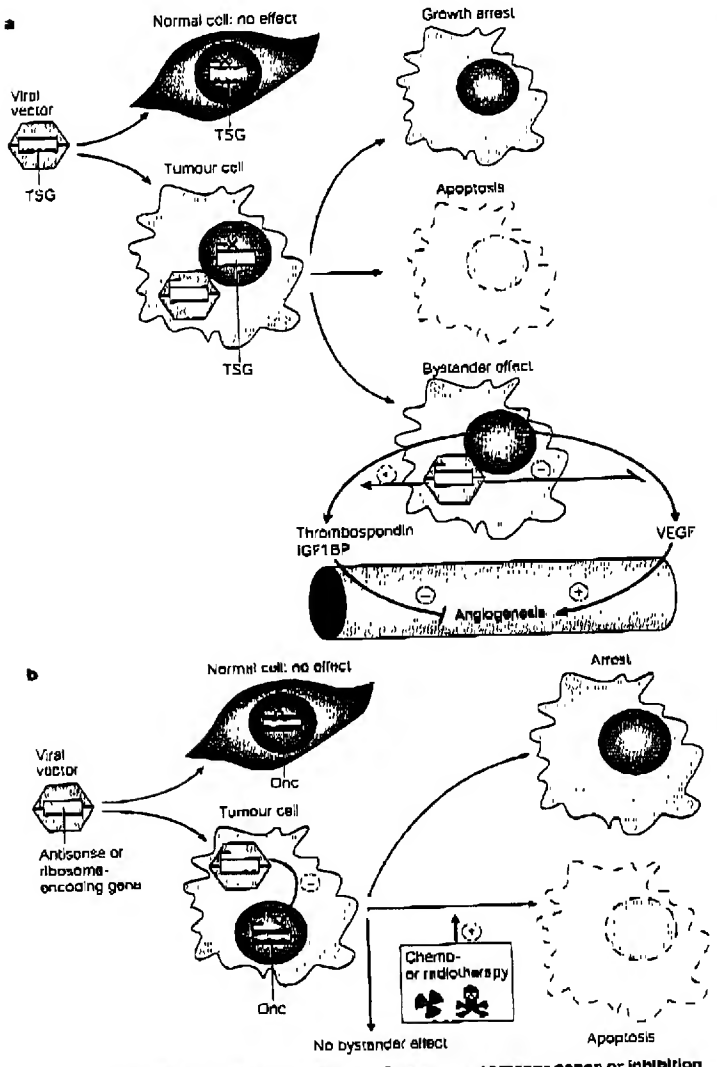

Figure 2 | **Cancer gene therapy by delivery of tumour-suppressor genes or inhibition of oncogene expression. a** | Tumour-suppressor gene (TSG) delivery. Vectors encoding the tumour suppressor of choice are assumed to infect normal cells and tumour cells. In tumour cells they induce either growth arrest or apoptosis, whereas in normal cells they are assumed not to have any detrimental effects. Some tumour suppressors might also exert unexpected bystander effects. For example, p53 blocks angiogenesis by downregulating the production of vascular endothelial growth factor (VEGF) and by upregulating two anti-angiogenic molecules, thrombospondin and insulin-like growth factor 1 binding protein (IGF1BP). **b** | Delivery of agents that block oncogene (Onc) expression. These include genes that encode antisense oligonucleotides, which block oncogene expression, and ribozymes, which cleave oncogene transcripts. Again, they are expected to have no detrimental effects on normal cells, which don't express oncogenes. By contrast, they should cause cancer cells to arrest or undergo apoptosis. In some cases, they also sensitize radio- or chemo-resistant tumour cells to radiotherapy or chemotherapy. No bystander effects have been reported for anti-oncogenic gene-therapy agents.

of IGF-1 and could kill uninfected tumour cells that depend on IGF-1 for survival. Yet another potential means of invoking a bystander effect is local inflammation triggered by proteins in the viral vector. These might stimulate production of cytokines, such as tumour necrosis factor, which might be able to kill uninfected tumour cells selectively[24]. These interpretations are hypothetical, but they illustrate the tremendous biological complexity encountered *in vivo* and show that gene-therapy agents can have profound biological effects that cannot be explained on the basis of their expected direct mechanism of action alone.

How does this approach fare in the clinic? The first clinical effects of delivering a tumour-suppressor gene — *TP53* — were published in 1996 (REF 11): RETROVIRAL VECTORS that express *TP53* from the actin promoter were injected directly into non small-cell lung tumours. Clear signs of apoptosis were detected in injected tumours, and three out of nine patients showed regression of these tumours, as well as evidence of a bystander effect. More recent trials have used ADENOVIRAL VECTORS to deliver *TP53* (Adp53), as these are easier to grow to high titres, and infect cells regardless of whether they are in the cell cycle. Adp53 is well tolerated and expressed in most patients. Furthermore, antitumour activity has been observed in a subset of patients who were treated for non-small-cell lung cancer[25] or squamous cell carcinoma of the head and neck[26]. Adp53 is now being tested in a Phase II and III clinical trial, as well as in combination with chemotherapeutic agents[27] or radiation.

If reactivating tumour suppressors is effective, what about blocking hyperactive oncogenes? Viral vectors have been used to deliver ANTISENSE OLIGONUCLEOTIDES or RIBOZYMES to block oncogene expression. The effectiveness of these approaches depends on similar assumptions to those of tumour-suppressor gene therapy, and when tested these assumptions have been fulfilled (TABLE 2): tumour cells with many genetic defects still depend on oncogene expression for growth or survival. For example, tumours that are driven by oncogenic *HRAS* are completely destroyed when *HRAS* is eliminated, using either genetic or pharmacological approaches[28,29], and chronic myelogenous leukaemias undergo dramatic remissions when the oncoprotein p210 BCR–ABL is blocked by the ABL inhibitor STI-571 (Gleevec), despite the presence of several genetic alterations in these cells[30]. Furthermore, ribozymes that target mutant RAS selectively should have no effect on normal cells. However, the viability of this approach might be hindered by lack of a bystander effect when tumour cells are treated in this way, and by the increasing number of alternative approaches to oncogene-directed therapy. Recent successes with small molecules, particularly inhibitors of kinases in the RAS pathway[31], p210 BCR–ABL and the epidermal growth factor receptor (EGFR)[32], indicate that more attention will be paid to this technology platform for oncogene-based therapy. By contrast, it is more difficult to develop small molecules that reactivate mutated tumour suppressors.

Table 2 | **Effects of blocking oncogenes in tissue culture and in mouse models**

Gene product	Function of oncogene	Strategy	Expression in cell lines	Expression in mouse models	References
ERBB2	Receptor tyrosine kinase activated by EGF	Ribozyme	Blocks cell proliferation	Inhibition of tumour growth	99–101
ERBB4	Receptor tyrosine kinase activated by neuregulins	Ribozyme	Growth inhibition	Inhibition of tumour growth	102
KRAS	Small GTP-binding protein; activates growth and survival pathways	Antisense; ribozyme	Growth inhibition	Tumour regression; apoptosis; chemosensitization	103–106
HRAS	Small GTP-binding protein; activates growth and survival pathways	Ribozyme	Growth inhibition	Tumour regression	107
HPV E6/E7	E7 inhibits RB; E6 targets p53 for destruction	Antisense; ribozymes	Not done	No effect	108
BCL2	Inhibits mitochondrial apoptosis pathway	Ribozyme	Reduces *BCL2* expression	Not done	109
Telomerase	Maintains telomere length to promote cellular immortality	Ribozyme	Chemosensitization	Not done	110
c-MET	Receptor tyrosine kinase activated by scatter factor	Ribozyme	Reduced migration; invasion	Not done	111
c-MYC	Transcription factor downstream of growth-factor signalling pathways	Ribozyme	Inhibits proliferation	Not done	112

EGF, epidermal growth factor; GTP, guanosine triphosphate; HPV, human papillomavirus; RB, retinoblastoma.

Exploiting bystander effects

The second broad approach to direct targeting of cancer cells with gene therapy is the delivery of suicide genes to cancer cells (FIG. 3). These are enzyme-encoding genes that, once expressed, allow the cancer cell to metabolize a harmless prodrug, administered separately, into a potent cytotoxin that can diffuse into neighbouring cells, so creating a bystander effect. Several enzyme–prodrug combinations are being evaluated (TABLE 3), but herpes simplex virus thymidine kinase (HSV-tk) has been most widely used and has progressed farthest into the clinic: many Phase I/II trials of this approach are ongoing in the United States, and one Phase III trial has been completed. HSV-tk converts ganciclovir to the phosphorylated form that becomes incorporated into DNA, thereby blocking DNA synthesis[10].

Selective expression of suicide genes could be achieved in several ways. Local injection of non-selective agents is an obvious solution. Early studies used retroviral vectors to deliver suicide genes to glioblastoma multiforme — a lethal form of brain tumour; these viruses are produced locally from injected PRODUCER CELLS. Retroviruses integrate only into dividing cells, so tumour cells growing in the brain express the transgene selectively. Local infusion or injection of nonspecific vectors (such as adenoviral vectors) directly into tumours also affords a degree of selectivity[24].

However, a more general strategy to kill tumour cells with suicide genes exploits tumour-specific expression elements. Many of these have been tested in cell systems and in animal models, and a fairly comprehensive repertoire of selective expression elements

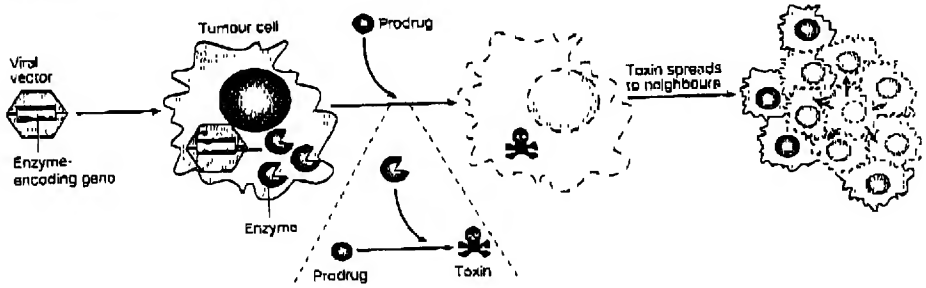

Figure 3 | **Suicide gene delivery.** The vector delivers a gene that encodes a prodrug-converting enzyme, such as herpes simplex virus thymidine kinase (HSV-tk), to tumour and normal cells alike. Local delivery of either the prodrug (in this case, ganciclovir) or the vector to the tumour provides specificity. The prodrug is converted to the active, cytotoxic metabolite in the tumour cell, and diffusion to neighbouring cells confers a potent bystander effect.

PRODUCER CELLS
Cells used to produce replication-defective viral vectors; they are transfected with the genes that are missing from the vector itself, and so provide the products of these genes in trans.

Table 3 | **Enzyme–prodrug combinations for suicide gene therapy***

Enzyme	Prodrug	Product	Mechanism	References
HSV-tk	Ganciclovir	Ganciclovir triphosphate	Blocks DNA synthesis	33
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil (5-FU)	Pyrimidine antagonist: blocks DNA and RNA synthesis	113,114
Nitroreductase	Nitrobenzyloxycarbonyl anthracyclines	Anthracyclines	DNA crosslinking	115
Carboxylesterase	CPT-11	SN38	Topoisomerase inhibitor	116
Cytochrome P450	Cyclophosphamide	Phosphoramide mustard	DNA alkylating agent: blocks DNA synthesis	117
Purine nucleoside phosphorylase	6-Mercaptopurine-DR	6-Mercaptopurine	Purine antagonist: blocks DNA synthesis	118

*Herpes simplex virus thymidine kinase (HSV-tk), in combination with the prodrug ganciclovir, is the most frequently used enzyme–prodrug combination for suicide gene therapy, but other approaches have been investigated. Cytosine deaminase (CD) is an effective alternative to HSV-tk, with a more substantial bystander effect[112]. Furthermore, local production of 5-FU by tumour-selective expression of CD sensitizes nearby tumour cells to radiation therapy[114]. Using adenovirus vectors, expression of CD suppresses growth of hepatic colon cancer metastases and increased survival significantly in mouse models[110–111]. Phase I clinical trials using CD targeted to breast cancer have shown that this approach is safe and relatively efficient at expressing CD in targeted tumours[122]. A Phase I study targeting metastatic colon cancer using CD is also underway[34].

is now available, TABLE 4 summarizes these elements and the principles that underlie their selectivity.

Bystander effects can also be achieved in other ways. Several strategies to suppress angiogenesis have been tested in animal models. For example, adenoviral expression of a soluble form of VEGF receptor was recently shown to suppress tumour growth in mouse models[35]. Angiostatin and endostatin expressed from plasmid DNA complexed with liposomes inhibited growth of breast cancer in mice[36]. An adenovirus expressing secretable endostatin showed activity *in vitro* and in mouse models[37], and a combination of viruses expressing three anti-angiogenic proteins led to complete tumour rejection in mouse models[38]. Another approach is to inhibit matrix metalloproteinases: the metalloproteinase inhibitor TIMP-2 expressed in an adenoviral vector caused significant reduction in metastatic cell growth[39]. These examples reflect the application of many years of discovery research in tumour biology to address clinical issues, and illustrate the translational opportunities that this field presents.

Clinical studies with suicide gene therapy have shown that these agents are safe, but are not sufficiently active. A Phase III clinical trial of retrovirus-encoded HSV-tk showed no patient benefit[40]. Local injection into brain tumours of adenoviral vectors that express HSV-tk looks slightly more promising, with some early signs of survival benefit in a small number of patients[41]. Nevertheless, there is an obvious need to improve efficacy. Efforts are underway to develop this concept further, using different vectors and suicide genes that might produce stronger bystander effects, combinations of suicide genes that act synergistically[42] and by pharmacological manipulation. For example, GAP JUNCTIONS can be upregulated by lovastatin and other compounds[43], increasing the magnitude of the bystander effect. Another problem is that many tissue-specific promoters are comparatively weak. Sakai and co-workers[44] have developed an innovative approach to solve this problem, by using a weak, but specific, promoter to drive expression of Cre recombinase, and a strong Cre-responsive promoter to drive expression of the therapeutic transgene.

These creative solutions will certainly improve the potency of suicide gene therapy, but will they be sufficient? Poor distribution of vectors within solid tumours and low infectivity of tumour cells might be the main barriers to success in this area. The next generation of protocols and vectors will need to include many innovations to improve selectivity and gene expression, but will also need to address these issues to realize their full clinical potential.

Replication-competent viruses

Exploitation of bystander effects represents one solution to the technical hurdle of infecting all the cells in a tumour. A second approach uses the ability of viruses to spread from their site of inoculation and infect neighbouring cells. In this approach, cells are killed as a consequence of virus infection, as they become factories for producing new infectious virus particles (FIG. 4). The success of this approach depends on our ability to engineer or select viruses that replicate specifically in tumour cells, and the ability of these viruses to infect tumour cells efficiently and to spread through the tumour.

The possibility of using viruses to kill cancer cells selectively was proposed and tested in 1956 (REF. 45), based on the observation that certain viruses grow efficiently in cancer cells, and on anecdotal reports of spontaneous tumour regressions in patients who harbour viral infections. Attempts to treat cervical cancer by injecting an adenovirus date back to 1956 (REF. 45), and the mumps virus was used to treat cancer in 1974 (REF. 45). However, lack of molecular characterization, of either the viruses or the tumours, prevented logical development of this approach. More recently, several viruses have been created that replicate selectively in cancer cells[45]. Indeed, this marriage of molecular virology and cancer biology provides a tremendous range of opportunities for creative engineering of selective agents. In retrospect, the field of tumour-virus biology sought to use viral oncoproteins to understand the molecular basis of cancer. Landmark discoveries in human cancer genetics, such as the functions of RB and p53, revealed the significance of the classical tumour-virus proteins — E1A and SV40 large T-antigen — in viral replication. Ironically, tumour viruses themselves are now at the forefront of cancer therapy.

GAP JUNCTION
A junction between two cells that allows the passage of molecules (up to 0 kDa).

Table 4 | **Regulated gene expression in tumour cells**

Regulatory element	Principle	References
Retrovirus LTR	Only expresses in dividing cells	34
Androgen receptor promoter	Prostate specific	123
Midkine promoter	Various tumours	124
VEGF promoter	Hypoxia specific	125
GRP promoter	SCLC specific	126
Kallikrein 2	Prostate specific	127,128
Osteocalcin	Osteotropic	129
TCF-4	Colorectal cancer (and others)	130
MUC1	Breast-cancer selective	70,131
TERT	Tumour selective	132
Thyroglobulin	Thyroid carcinoma	133
MYC–MAX motif	SCLC specific	134
Surfactant protein B	Alveolar, bronchial cancers	65
α-Fetoprotein	Hepatocellular carcinoma	44
CEA promoter	Gastrointestinal cancer	135,136
Tyrosinase promoter	Melanoma specific	137
Fibrillary acidic protein	Glial-cell specific	138
E-selectin promoter	Endothelial-cell specific	139
EGR-1 promoter	Glial-cell selective	140
PSA promoter	Prostate specific	141
GFAP promoter	Astrocyte specific	142
E2F1	Tumour selective	143
Myelin basic promoter	Malignant glioma	144
von Willebrand factor promotor	Endothelial cells	145
α-Lactalbumin	Breast cancer	146

CEA, carcinoembryonic antigen; EGR-1, early growth-responsive 1; GFAP, glial fibrillary acid protein; GRP, gastrin-related peptide; LTR, long terminal repeat; MUC1, mucin 1; PSA, prostate-specific antigen; SCLC, small-cell lung cancer; TCF-4, T-cell factor 4; TERT, human telomerase reverse transcriptase; VEGF, vascular endothelial growth factor.

Targeting dividing cells with herpesviruses. The first engineered therapeutic virus to enter clinical testing was a defective herpesvirus mutant, G207, that lacks the gene encoding ribonucleotide reductase[48] and can therefore replicate efficiently only in dividing cells. G207 also lacks the virulence gene *ICP34.5*, to improve safety. This virus was initially tested in mouse models by direct injection into tumours in the brain. On the basis of encouraging data from these preclinical experiments, G207 advanced to a Phase I clinical trial for malignant glial-cell tumours. G207 administered directly into these tumours was safe and well-tolerated, and early signs of clinical effects have been reported[47]. In parallel, another *ICP34.5* mutant, HSV-1716, has undergone successful preclinical testing for metastatic melanoma[48] and has recently been injected directly into subcutaneous lesions of metastatic melanoma[49] and into recurrent malignant gliomas[50] in Phase I clinical trials. So far, these agents seem to be safe and well tolerated.

Targeting a defective p53 pathway. An attenuated adenovirus — mutant dl1520 (REF. 51) or ONYX-015 — relies on a coincidental biological property of adenoviruses and tumour cells: both need to block p53 function to replicate efficiently (FIG. 5)[52]. Adenoviruses eliminate p53 by producing the EARLY REGION PROTEIN E1B 55K. This protein binds p53 and, with help from another viral protein, E4 orf6, targets it for destruction. ONYX-015 lacks E1B 55K and therefore cannot destroy p53. In normal cells, p53 blocks replication, but in cancer cells that lack p53, ONYX-015 can replicate — at least in theory.

The specificity of ONYX-015 for p53-deficient cells remains controversial[53]. Confusion has arisen for two reasons: first, ONYX-015 can replicate in some tumour cells that retain wild-type p53, apparently contradicting the premise of its specificity[54–57]. However, most tumour cells that retain wild-type p53 have other defects in the p53 pathway (FIG. 5). Assessment of the functional status of p53 based solely on the presence or absence of mutant p53 is therefore misleading. In normal cells, MDM2, an E3 UBIQUITIN LIGASE that targets p53 for destruction, keeps the levels of p53 low. Loss of ARF, a protein that inhibits MDM2, is a common mechanism of suppressing p53. In tumour cells that lack ARF, p53 is not activated during ONYX-015 infection, and therefore cannot block replication. Expression of *ARF* in such cells induces high levels of p53 that inhibits replication of ONYX-015, but not wild-type adenovirus replication[58].

The second source of confusion derives from the fact that E1B 55K has other functions that are distinct from blocking p53. These functions affect selective export and translation of viral mRNAs during infection (reviewed in REF. 59). Some tumour cells (such as C33a cervical carcinoma[52]) complement these other functions of E1B 55K perfectly, and others (such as U2OS osteosarcoma[53]) do not. Among tumours with a defective p53 pathway, then, variations in ONYX-015 replication are determined by the degree to which these other functions of E1B 55K are complemented[60].

While these issues regarding the selectivity of ONYX-015 were being investigated, clinical development of ONYX-015 proceeded. Treatment of patients with head and neck cancer — who had failed surgery, radiation and chemotherapy — with ONYX-015 alone revealed that this agent is safe and well tolerated, but clinical responses were modest[61]. Responses might have been limited by several factors, but poor distribution of injected virus seems likely to be the most significant limitation. Tumours of this type, particularly those from patients treated by radiation, contain high proportions of stromal and fibrotic tissue that are likely to limit efficient spread of virus through the tumour.

Higher response rates were seen in patients with recurrent head and neck cancer treated with ONYX-015 in combination with 5-fluorouracil and cisplatin[61], and a Phase III trial is now in progress. In these trials, evidence has accumulated that a bystander effect contributes to the clinical outcome. Anecdotally, tumours injected with ONYX-015 show widespread necrosis in a timeframe that cannot be accounted for by viral replication alone. Virus replication triggers an acute and local inflammatory reaction that involves production of tumour necrosis factor-α and other cytokines.

EARLY REGION PROTEIN
Viral proteins expressed before the onset of viral DNA synthesis, usually involved in driving the infected cell into the S-phase of the cell cycle.

E3 UBIQUITIN LIGASE
The third enzyme in a series — the first two are designated E1 and E2 — that are responsible for ubiquitylation of target proteins. E3 enzymes provide platforms for binding E2 enzymes and specific substrates, thereby coordinating ubiquitylation of the selected substrates.

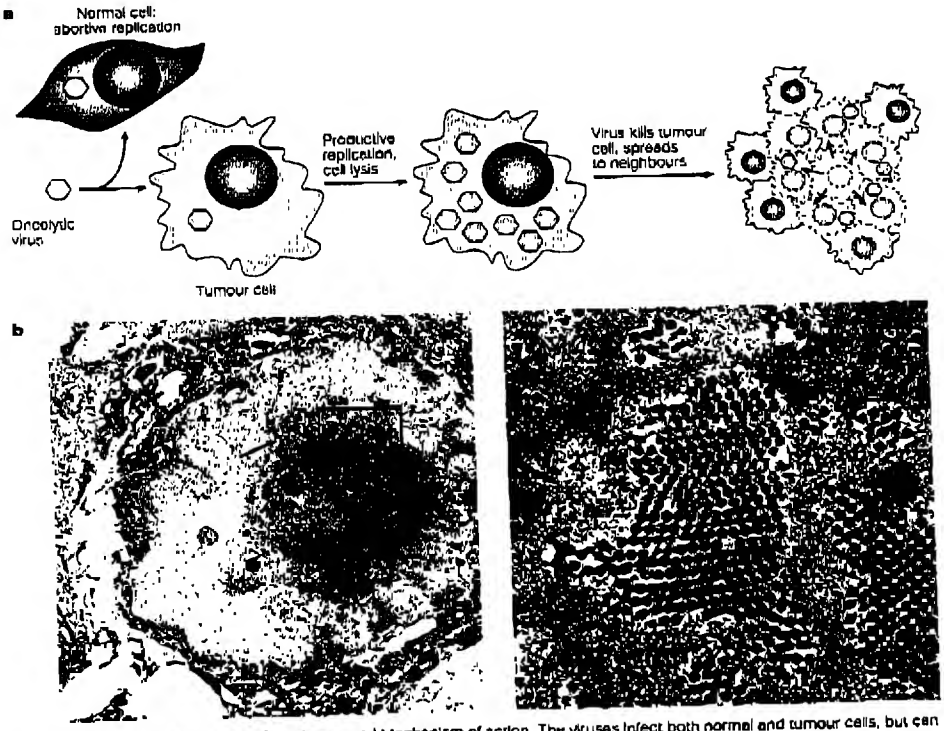

Figure 4 | **Conditionally replicating viruses.** **a** | Mechanism of action. The viruses infect both normal and tumour cells, but can only replicate in tumour cells. The progeny then go on to kill surrounding tumour cells. **b** | Replication of a conditionally replicating virus, ONYX-015, in a cancer cell from a patient with head and neck cancer during Phase II clinical testing. 10^{12} infectious particles were injected over a 5-day period. After 8 days, biopsy was performed and analysed by electron microscopy. The inset on the left panel is magnified on the right. Clearly, this cell is doomed to die: presumably the new virus particles it produces will infect its neighbours.

These agents might kill nearby tumour cells with some selectivity, augmented by the presence of chemotherapeutic agents. Ironically, this bystander effect might be more effective in tumours that retain wild-type p53. The overall clinical outcome of this treatment therefore depends on complicated interactions between the host immune system, the tumour cell and its neighbours, and the replicating virus.

Targeting the RB pathway. Adenovirus E1A proteins bind and neutralize RB and its relatives. This releases the transcription factor E2F, which is repressed by RB, with consequences for both the virus and its host. The cellular function of E2F is to drive the expression of genes involved in S-phase and chromatin synthesis, but adenovirus has also hijacked E2F to activate expression of the E2 region of the viral genome. A virus expressing a mutant form of E1A that fails to bind RB should therefore be restricted to cells that lack functional RB. Most, if not all, tumour cells are defective in the RB pathway and, as a result, should be permissive to E1A-defective viruses. Such viruses have been described[82–84] and tested in preclinical models. The potency of one of these viruses can be increased further by engineering it to overexpress the adenovirus death protein (ADP): this increases cell lysis and virus release, making the virus more active *in vivo* than the E1A mutant alone[85]. E1A has many additional functions: it binds to p300 and it activates the E4 region of the viral genome[44]. Viruses that exploit these features are under development[84] (L. Johnson, F. M. and A. Fattaey, unpublished observations).

Tissue or tumour-specific regulation of viral replication. Viruses that replicate in cancer cells or tissues selectively based on differential regulation of viral gene expression have also been engineered and tested in the clinic. For example, Celydon CN706 (REF. 87) is an adenovirus in which the prostate-specific antigen (PSA) promoter drives E1A. In CN787, the rat probasin promoter (another prostate-specific regulatory element) drives E1A and the PSA-regulatory element drives E1B (REF. 88). These

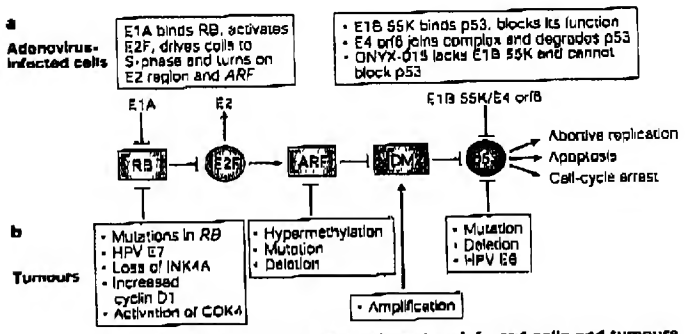

Figure 5 | **Alterations in the p53 pathway in adenovirus-infected cells and tumours.** a | Adenovirus E1A binds the tumour suppressor RB, thereby preventing it from repressing E2F. E2F is then free to activate host target genes that are involved in S-phase of the cell cycle, and the adenovirus E2 region. One of the host genes that E2F activates is *ARF*, an inhibitor of the oncoprotein MDM2. MDM2 is an E3 ubiquitin ligase that targets the tumour suppressor p53 for destruction. Adenovirus E1B 55K and E4 ORF6 cooperate to target p53 for destruction, because in the presence of p53 viral replication cannot occur. b | In tumours, there are several mechanisms by which the RB and p53 pathways can be inactivated. RB is mutated in approximately 25% of tumours, but can also be functionally inactivated by over-activation of
D-type cyclins, which phosphorylate and inactivate RB. This can occur by loss of the CDK inhibitor INK4A, increased expression of cyclin D1 or activation of CDK4. In addition, the human papillomavirus (HPV) E7 protein, similar to adenovirus E1A, can bind to and inhibit RB. p53 itself is mutated or deleted in around 60% of tumours, and can be targeted for destruction by HPV E6 protein. It can also be functionally inactivated by amplification of MDM2, or inactivation of ARF by hypermethylation, mutation or deletion.

viruses do not discriminate between normal and cancerous prostate because both PSA and probasin are produced by normal prostate tissue, but in this clinical setting this is not thought to be a significant issue. Both viruses are currently undergoing clinical investigation.

Iggo and co-workers[69] have made a virus that depends on abnormal signalling from the β-catenin/TCF-4 pathway to replicate selectively in colon cancer cells. Virtually all colorectal cancer cells, and many other types of cancers, have a defect in this pathway. Loss of the tumour suppressor APC in up to 80% of colorectal cancers allows accumulation of high levels of β-catenin, because one function of APC is to target β-catenin for destruction. In other tumours, mutations occur in β-catenin itself that make it resistant to degradation. High levels of β-catenin bind to the transcription factor TCF-4, and binding derepresses TCF-responsive genes, such as cyclin D1, c-MYC and matrilysin. A virus containing a TCF-responsive element therefore grows selectively in cells in which this pathway is deregulated. Using a similar strategy, The MUC1 promoter has been used to drive E1A, so supporting virus replication selectively in breast cancer cells in which MUC1 is aberrantly expressed[70].

In many ways, this innovative technology is an extension of suicide gene therapy, and again illustrates that cancer gene therapy provides a field day for molecular biologists who are interested in applying basic principles of cancer biology to clinical problems. Unfortunately, commercial and clinical development of the viruses and vectors using tissue-specific promoters might be complicated by a dominating patent on the use of tissue-specific promoters for gene therapy (Novartis US patent 5,998,205, Hallenbeck, Chang and Chiang[71]).

Second-generation replicating viruses might combine these two strategies. For example, a virus similar to ONYX-015 that expresses HSV-tk seems to be more potent than either approach on its own[72,73]. Likewise, a replication-competent herpesvirus vector expressing HSV-tk in combination with a cytochrome P450 enzyme that converts the prodrug cyclophosphamide to its active phosphoramide derivative showed potent anti-tumour activity in mouse models[74].

Many viruses have a natural tropism for tumour cells, for one reason or another. RNA viruses such as Newcastle disease virus and vesicular stomatitis virus replicate in tumour cells because these cells fail to mount a protective interferon response. Parvoviruses, such as adenovirus-associated virus (AAV), replicate selectively in tumour cells because in normal cells, single-stranded AAV genomes trigger a p53-dependent DNA-damage response that blocks cells in G2 and prevents replication[75] — and for other reasons that are not fully understood. Another type of parvovirus, H1, has entered clinical testing based on its tumour selectivity. The merits of these approaches, and the status of clinical testing, have been discussed elsewhere[76].

Challenges

Clinical data from gene-therapy agents and replication-competent viruses administered locally indicate that some of these approaches will have clinical value, and indeed a significant number of patients could benefit from local or regional treatment. These include patients suffering from head and neck cancer, glioblastoma, ovarian cancer and metastatic colorectal cancer, which usually localizes to the liver. Two main challenges need to be overcome to convert cancer gene therapy into a strategy that is effective against disseminated metastatic disease. First, the vector or virus needs to reach the tumour efficiently. Second, it needs to avoid neutralization by the immune system. In contrast to more traditional approaches to cancer therapy, based on small-molecule inhibitors of enzyme targets, the success of this field lies in the hands of creative molecular biologists, rather than medicinal chemists and pharmacologists. These challenges might not have captured the imagination of basic scientists, but they are of paramount importance to successful evolution of the field and, again, present opportunities for state-of-the-art translational research.

Biodistribution. The magnitude of these problems is difficult to estimate accurately, but it is already clear that several issues need to be addressed. Agents that are administered systemically can interact with many cell types in the blood and with endothelial cells, and are cleared rapidly from the bloodstream by the liver[77]. If they survive these obstacles, they must leak from blood vessels into tumours, and spread within the heterogeneous mass of the tumour. These issues sound formidable, but, of course, they have been faced many times before, as all forms of systemic cancer therapies face similar problems.

MUC1
A large, transmembrane glycoprotein of the mucin family. It is often expressed on cancer cells, especially breast cancer cells.

A principal distinction is that for these biological agents, biologists might have the answers. For example, the adenovirus receptor, CAR[78], is an adhesion protein[79] that is expressed at high levels in liver, kidney, brain, heart, pancreas, endothelium, colon and prostate, but not in peripheral lymphocytes, spleen, skeletal muscle and fibroblasts[80]. Some advanced cancer cell lines lose CAR expression[81,82]. This information helps determine which normal cells are likely to be infected during systemic administration, and which tumour cells are susceptible. Tumour types that fail to express CAR should probably be excluded from trials using the current generation of adenovirus vectors.

Of greater significance for the long-term development of these vectors are intense efforts to re-direct adenoviruses away from CAR in favour of tumour cells. This has been facilitated by the rapid solution of the structure of CAR that is bound to adenovirus fibre protein[83]. One approach to re-targeting adenoviruses is to engineer ligands into viral proteins, and to use tumour cell-surface proteins (the fibroblast growth-factor receptor, for example[84]) as new receptors. Another is to increase binding of virus particles to integrins or simply to enhance CAR-independent non-specific attachment[85]. Another creative approach uses an adenovirus serotype that does not use CAR as its receptor, such as serotype 35 (REF. 86). Binding of CAR could also be masked by polyethylene glycol treatment of virus particles[77,87]. Alternatively, CAR expression might be regulated in tumour cells by pharmacological intervention (M. Anders, F. M. and W.M. Korn, unpublished observations). The full potential of gene therapy using adenoviruses might ultimately depend on one of these new strategies to infect tumour cells selectively, or prevent infection of normal cells that express relatively high levels of receptor. It is therefore likely that distribution of adenoviruses between normal cells and tumours can be changed dramatically by rational and creative molecular approaches, many of which are currently under active investigation.

Avoiding the humoral immune system. In other fields of gene therapy (cystic fibrosis or other heritable disorders, for example), persistent gene expression is often thwarted by destruction of the producer cell by both the B-cell and T-cell arms of the immune system. For cancer gene therapy, destruction of the target cell is an objective and, for this indication, suppression of the B-cell arm is the main issue (although the innate immune system can also contribute to virus neutralization[88]). Most adults have low levels of neutralizing antibodies against adenoviruses of the serotypes used in most contemporary vectors (Ad serotype 5), but for local injection or infusions of high doses of virus (10^{12} virions, for example), these levels of neutralizing antibody are probably insignificant. However, systemic treatment results in relatively low levels of circulating virus, and neutralizing antibodies are almost certain to curtail the effectiveness of these approaches, particularly as titres rise during repeated treatment[89]. Again, creative solutions might be at hand. Serotypes could be used that are less antigenic or less common in the population, and epitopes could be engineered out of viral particles or masked with agents such as polyethylene glycol[87]. Alternatively, the B-cell arm of the immune system could be suppressed by selective immune suppression, using the anti-CD20 antibody Rituxan, for example (T. Ried, personal communication). Whether these or other solutions are effective remains to be seen. A great deal depends on their success, as the future of gene therapy for systemic diseases would be transformed if this hurdle were eliminated or minimized.

Future prospects

The future of gene therapy for cancer depends on a combination of applied bioengineering and traditional clinical development. To a large extent, the problem of selectively killing cancer cells has been solved. Critics of cancer gene therapy would probably agree that the biological principles are sound, but translating these principles into reality remains a formidable — perhaps prohibitive — challenge. Most of the innovative strategies designed to circumvent these problems are still in preclinical or early clinical testing, and it is too early to predict how successful they will be. However, even the first-generation agents that have entered clinical testing have proved safe and there have been several suggestions of efficacy. Considering the small number of concepts that have been tested extensively (HSV-tk-based suicide vectors, Adp53, ONYX-015, G207 and CN706), it is too early to assess this field conclusively, particularly as only one of these approaches — HSV-tk-based suicide-gene therapy — has completed a Phase III trial.

The impact of cancer gene therapy will depend to some extent on the success of other strategies. Of these, small molecules that are directed at known molecular targets, and also therapeutic antibodies, seem to be the most promising. Small molecules are the most traditional and preferred approach for most pharmaceutical companies. However, this approach has so far been remarkably unsuccessful in oncology. Very few new small molecules have been approved for cancer treatment based on rational targets, despite tremendous efforts throughout the industry. However, the tide could be turning. With the dramatic results of STI-571 (Gleevec) in treating early stages of chronic myelogenous leukaemia, and promising data from epidermal growth-factor receptor (EGFR) inhibitors and others, a new era of optimism has arrived. But the problems of obtaining selectivity for cancer cells, and of achieving suitable biodistribution and safety, are formidable, and are frequently underestimated by investigators unfamiliar with the process of drug development. Furthermore, new agents such as Gleevec have already encountered the old problem of drug resistance that limits the potency of previous generations of small-molecule drugs[90]. Yet the success of Gleevec shows us that there is a successful path to a systemically active and safe anticancer drug, however tortuous this path may be. The same cannot yet be said of cancer gene therapy, even though the concepts have strong biological credentials.

HUMANIZED MONOCLONAL ANTIBODY
An antibody, usually from a rodent, engineered to contain mainly human sequences. This process reduces the immune response to the antibody in humans.

Antibody agents have enjoyed a renaissance. Indeed, trastuzumab (Herceptin), which blocks the oncogenic EGFR ERBB2, was the first approved drug to be developed directly as a result of the discovery of an oncogene, and similar agents are in the pipeline. Antibodies have more predictable pharmacological and toxicological properties than small molecules, and HUMANIZED versions are not as immunogenic as viral vectors, so do not face the problem of neutralization that must be addressed for cancer gene therapy. However, their impact is limited by the small number of targets that are accessible and specific on the surfaces of tumour cells.

Ultimately, these diverse approaches will be used together, as they use different mechanisms to control or kill cancer cells and they are very likely to have distinct toxicity profiles. In the early 1990s, it was not possible to claim with any assurance that cancer cells could be killed selectively and rationally. Now, the debate has shifted to a discussion of which approach is most likely to be successful and, in the case of cancer gene therapy, whether the ingenuity of bioengineers and clinical scientists can surmount the final hurdles. We already know how to make more potent suicide genes, and how to make viruses that replicate more efficiently in tumour cells. We can re-target viruses away from their natural receptors, and we can imagine ways to keep the immune system from attacking those therapeutic agents. None of these new ideas has yet been tested in the clinic, but any one of them could help enormously. It seems unlikely to me that cancer gene therapy will fall at the final hurdle.

1. Morin, P. J., Vogelstein, B. & Kinzler, K. W. Apoptosis and APC in colorectal tumorigenesis. *Proc. Natl Acad. Sci. USA* **93**, 7950–7954 (1996).
2. Nikitin, A. Y., Juarez-Perez, M. I., Li, S., Huang, L. & Lee, W. H. RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/− mice. *Proc. Natl Acad. Sci. USA* **96**, 3916–3921 (1999).
3. Demers, G. W. *et al.* A recombinant adenoviral vector expressing full-length human retinoblastoma susceptibility gene inhibits human tumor cell growth. *Cancer Gene Ther.* **5**, 207–214 (1998).
4. Craig, C. *et al.* Effects of adenovirus-mediated *p16*^{INK4} expression on cell cycle arrest are determined by endogenous p16 and Rb status in human cancer cells. *Oncogene* **16**, 265–272 (1998).
5. Sumitomo, K., Shimizu, E., Shinohara, A., Yokota, J. & Sone, S. Activation of RB tumor suppressor protein and growth suppression of small cell lung carcinoma cells by reintroduction of *p16*^{INK4} gene. *Int. J. Oncol.* **14**, 1075–1080 (1999).
6. Tanaka, M. *et al.* MMAC1/PTEN inhibits cell growth and induces chemosensitivity to doxorubicin in human bladder cancer cells. *Oncogene* **19**, 5406–5412 (2000).
7. Minaguchi, T. *et al.* Growth suppression of human ovarian cancer cells by adenovirus-mediated transfer of the *PTEN* gene. *Cancer Res.* **59**, 6063–6067 (1999).
8. Sakurada, A. *et al.* Adenovirus-mediated delivery of the *PTEN* gene inhibits cell growth by induction of apoptosis in endometrial cancer. *Int. J. Oncol.* **15**, 1069–1074 (1999).
9. Cheney, I. W. *et al.* Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated *MMAC1/PTEN* gene transfer. *Cancer Res.* **58**, 2331–2334 (1998).
10. Yang, C. T. *et al.* Adenovirus-mediated *p14*^{ARF} gene transfer in human mesothelioma cells. *J. Natl Cancer Inst.* **92**, 636–641 (2000).
11. Roth, J. A. *et al.* Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nature Med.* **2**, 985–991 (1996).
First report of p53 delivery to cancer cells in humans. Expression of p53 and increased apoptosis in treated tumours was reported, with no evidence of toxicity. The retrovirus delivery system was not sufficiently efficient, but this paper proved the principle that delivery of p53 could kill cancer cells *in vivo*.
12. Seth, P. *et al.* A recombinant adenovirus expressing wild type p53 induces apoptosis in drug-resistant human breast cancer cells: a gene therapy approach for drug-resistant cancers. *Cancer Gene Ther.* **4**, 383–390 (1997).
13. Holt, J. T. *et al.* Growth retardation and tumour inhibition by BRCA1. *Nature Genet.* **12**, 298–302 (1996).
14. Shao, N., Chai, Y. L., Shyam, E., Reddy, P. & Rao, V. N. Induction of apoptosis by the tumor suppressor protein BRCA1. *Oncogene* **13**, 1–7 (1996).
15. Tait, D. L. *et al.* A phase I trial of retroviral *BRCA1sv* gene therapy in ovarian cancer. *Clin. Cancer Res.* **3**, 1959–1968 (1997).
16. Vogelstein, B., Lane, D. & Levine, A. J. Surfing the p53 network. *Nature* **408**, 307–310 (2000).
17. Kaplan, K. B. *et al.* A role for the adenomatous polyposis coli protein in chromosome segregation. *Nature Cell Biol.* **3**, 429–432 (2001).
18. Fodde, R. *et al.* Mutations in the *APC* tumour suppressor gene cause chromosomal instability. *Nature Cell Biol.* **3**, 433–438 (2001).
19. Zhang, W. W. *et al.* High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther.* **1**, 5–13 (1994).
20. Nishizaki, M. *et al.* Recombinant adenovirus expressing wild-type p53 is antiangiogenic: a proposed mechanism for bystander effect. *Clin. Cancer Res.* **5**, 1015–1023 (1999).
21. Bouvet, M. *et al.* Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. *Cancer Res.* **58**, 2288–2292 (1998).
22. Dameron, K. M., Volpert, O. V., Tainsky, M. A. & Bouck, N. The p53 tumor suppressor gene inhibits angiogenesis by stimulating the production of thrombospondin. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 483–489 (1994).
First connection between p53 and angiogenesis: the discovery that p53 regulates expression of the production of thrombospondin, a protein purified by Bouck and co-workers in an early biological assay for factors that block angiogenesis.
23. Buckbinder, L. *et al.* Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377**, 646–649 (1995).
24. Mueller, H. Tumor necrosis factor as an antineoplastic agent: pitfalls and promises. *Cell. Mol. Life Sci.* **54**, 1291–1298 (1998).
25. Swisher, S. G. *et al.* Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J. Natl Cancer Inst.* **91**, 763–771 (1999).
26. Clayman, G. L., Frank, D. K., Bruso, P. A. & Goepfert, H. Adenovirus-mediated wild-type p53 gene transfer as a surgical adjuvant in advanced head and neck cancers. *Clin. Cancer Res.* **5**, 1715–1722 (1999).
27. Nemunaitis, J. *et al.* Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J. Clin. Oncol.* **18**, 609–622 (2000).
28. Chin, L. *et al.* Essential role for oncogenic Ras in tumour maintenance. *Nature* **400**, 468–472 (1999).
29. Barrington, R. E. *et al.* A farnesyltransferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. *Mol. Cell Biol.* **18**, 85–92 (1998).
30. Efficacy and safety of a specific inhibitor of the BCR–ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* **344**, 1031–1037 (2001).
One of a series of landmark papers from Druker and colleagues, showing the clinical effects of STI-571 (Gleevec) in clinical trials. First successful demonstration that small molecules that target oncogenes can have clinical benefit.
31. Sebolt-Leopold, J. S. *et al.* Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nature Med.* **5**, 810–816 (1999).
32. Mendelsohn, J. & Baselga, J. The EGF receptor family as targets for cancer therapy. *Oncogene* **19**, 6550–6565 (2000).
33. Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.* **46**, 5276–5281 (1986).
Early description of the concept of suicide gene therapy, in which HSV-tk is expressed in cells using viral vectors, and cells and their uninfected neighbours are killed by the approved clinical prodrug, ganciclovir. This system has been the most widely used of all suicide gene-therapy protocols.
34. Crystal, R. G. *et al.* Phase I study of direct administration of a replication deficient adenovirus vector containing the *E. coli* cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum. Gene Ther.* **8**, 985–1001 (1997).
35. Takayama, K. *et al.* Suppression of tumor angiogenesis and growth by gene transfer of a soluble form of vascular endothelial growth factor receptor into a remote organ. *Cancer Res.* **60**, 2169–2177 (2000).
36. Chen, Q. R., Kumar, D., Stass, S. A. & Mixson, A. J. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res.* **59**, 3308–3312 (1999).
37. Chen, C. T. *et al.* Antiangiogenic gene therapy for cancer via systemic administration of adenoviral vectors expressing secretable endostatin. *Hum. Gene Ther.* **11**, 1983–1996 (2000).
38. Regulier, E. *et al.* Adenovirus-mediated delivery of antiangiogenic genes as an antitumor approach. *Cancer Gene Ther.* **8**, 45–54 (2001).
39. Brand, K. *et al.* Treatment of colorectal liver metastases by adenoviral transfer of tissue inhibitor of metalloproteinases-2 into the liver tissue. *Cancer Res.* **60**, 5723–5730 (2000).
40. Rainov, N. G. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum. Gene Ther.* **11**, 2389–2401 (2000).
41. Sandmair, A. M. *et al.* Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum. Gene Ther.* **11**, 2197–2205 (2000).
42. Aghi, M., Kramm, C. M., Chou, T. C., Breakefield, X. O. & Chiocca, E. A. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J. Natl Cancer Inst.* **90**, 370–380 (1998).
43. Touraine, R. L., Vahanian, N., Ramsey, W. J. & Blaese, R. M. Enhancement of the herpes simplex virus thymidine kinase/ganciclovir bystander effect and its antitumor efficacy *in vivo* by pharmacologic manipulation of gap junctions. *Hum. Gene Ther.* **9**, 2385–2391 (1998).
44. Sakai, Y. *et al.* Gene therapy for hepatocellular carcinoma using two recombinant adenovirus vectors with α-fetoprotein promoter and Cre/lox P system. *J. Virol. Methods* **92**, 5–17 (2001).
45. Kirn, D. H. & McCormick, F. Replicating viruses as selective cancer therapeutics. *Mol. Med. Today* **2**, 519–527 (1996).
46. Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D. & Martuza, R. L. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nature*

Med. 1, 938–943 (1995)
First use of a recombinant conditionally replicating virus to treat human cancer. This HSV mutant cannot replicate in quiescent cells because a key enzyme involved in DNA synthesis has been deleted. This mutant virus kills brain tumour cells selectively, because normal cells cannot support replication.

47. Markert, J. M. *et al.* Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther.* 7, 867–874 (2000)
48. Randazzo, B. P., Bhat, M. G., Kesari, S., Fraser, N. W. & Brown, S. M. Treatment of experimental subcutaneous human melanoma with a replication-restricted herpes simplex virus mutant. *J. Invest. Dermatol.* 108, 933–937 (1997).
49. MacKie, R. M., Stewart, B. & Brown, S. M. Intralesional injection of herpes simplex virus 1716 in metastatic melanoma. *Lancet* 357, 525–526 (2001).
50. Rampling, R. *et al.* Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.* 7, 859–866 (2000).
51. Barker, D. D. & Berk, A. J. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156, 107–121 (1987).
52. Bischoff, J. R. *et al.* An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 274, 373–376 (1996).
First virus described for which replication depends on loss of p53. This adenovirus mutant grows selectively in tumour cells in which p53 is inactive, but also depends on other functions of tumour cells that are not fully understood for its efficient replication. In this paper, it was shown for the first time that a recombinant virus destroys human xenograft tumours in mouse models of cancer.
53. Dix, B. R., Edwards, S. J. & Braithwaite, A. W. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? *J. Virol.* 75, 5443–5447 (2001).
54. Heise, C. C., Williams, A., Olesch, J. & Kirn, D. H. Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: intratumoral spread and distribution effects. *Cancer Gene Ther.* 6, 499–504 (1999).
55. Goodrum, F. D. & Ornelles, D. A. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J. Virol.* 72, 9479–9490 (1998)
56. Rothmann, T., Hengstermann, A., Whitaker, N. J., Scheffner, M. & zur Hausen, H. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J. Virol.* 72, 9470–9478 (1998)
57. Turnell, A. S., Grand, R. J. A. & Gallimore, P. H. The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status. *J. Virol.* 73, 2074–2083 (1999)
58. Ries, S. J. *et al.* Loss of $p14^{ARF}$ in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). *Nature Med.* 6, 1128–1133 (2000)
59. Harada, J. N. & Berk, A. J. p53-independent and -dependent requirements for E1B-55K in adenovirus type 5 replication. *J. Virol.* 73, 5333–5344 (1999)
60. Nemunaitis, J. *et al.* Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J. Clin. Oncol.* 19, 289–298 (2001).
61. Khuri, F. R. *et al.* A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nature Med.* 6, 879–885 (2000)
62. Heise, C. *et al.* An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nature Med.* 6, 1134–1139 (2000)
63. Fueyo, J. *et al.* A mutant oncolytic adenovirus targeting the RB pathway produces anti-glioma effect in vivo. *Oncogene* 19, 2–12 (2000).
A mutant adenovirus that cannot neutralize RB is shown to replicate selectively in cancer cells. This approach is similar to the approach of dl1520, ONYX-015. In this case, a small mutation was made to inactivate E1A's ability to neutralize RB, and other functions of E1A were left intact.
64. Doronin, K. *et al.* Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J. Virol.* 74, 6147–6155 (2000).
65. Doronin, K. *et al.* Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J. Virol.* 75, 3314–3324 (2001)
66. Nevins, J. R. Adenovirus E1A-dependent trans-activation of transcription. *Semin. Cancer Biol.* 1, 59–68 (1990)
Description of E2F, the crucial transcription factor activity responsible for entry into S-phase of the cell cycle. This factor is misregulated in almost all cancers, and is also vital for adenovirus replication, through its effects on S-phase and on activation of the E2 region of the viral genome.
67. Rodriguez, R. *et al.* Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 57, 2559–2563 (1997).
68. Yu, D. C. *et al.* Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res.* 61, 517–525 (2001).
69. Brunori, M., Malerba, M., Kashiwazaki, H. & Iggo, R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. *J. Virol.* 75, 2857–2865 (2001)
70. Kurihara, T., Brough, D. E., Kovesdi, I. & Kufe, D. W. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J. Clin. Invest.* 106, 763–771 (2000)
71. Hallenbeck, P. L., Chang, Y.-N. & Chiang, Y. L. Vectors for tissue-specific replication. US Patent 5,998,205 (1999).
72. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D. & Kim, J. H. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum. Gene Ther.* 9, 1323–1333 (1998).
73. Wildner, O. *et al.* Adenoviral vectors capable of replication improve the efficacy of *HSVtk/GCV* suicide gene therapy of cancer. *Gene Ther.* 6, 57–62 (1999)
74. Aghi, M., Chou, T. C., Suling, K., Breakefield, X. O. & Chiocca, E. A. Multimodal cancer treatment mediated by a replicating oncolytic virus that delivers the oxazaphosphorine/rat cytochrome P450 2B1 and ganciclovir/herpes simplex virus thymidine kinase gene therapies. *Cancer Res.* 59, 3861–3865 (1999)
75. Raj, K., Ogston, P. & Beard, P. Virus-mediated killing of cells that lack p53 activity. *Nature* 412, 914–917 (2001)
76. Kirn, D., Martuza, R. L. & Zwiebel, J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nature Med.* 7, 781–787 (2001)
77. Alemany, R., Suzuki, K. & Curiel, D. T. Blood clearance rates of adenovirus type 5 in mice. *J. Gen. Virol.* 81, 2605–2609 (2000).
78. Bergelson, J. M. *et al.* The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J. Virol.* 72, 415–419 (1998)
Identification of the cellular receptor for adenovirus, using an ingenious expression-cloning strategy. Identification of CAR has greatly increased our understanding of adenovirus infectivity and efficiency of vectors using adenoviruses to deliver genes or kill cells through replication.
79. Honda, T. *et al.* The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. *Brain Res. Mol. Brain Res.* 77, 19–28 (2000).
80. Fechner, H. *et al.* Expression of coxsackie adenovirus receptor and α-integrin does not correlate with adenovector targeting in vivo indicating anatomical vector barriers. *Gene Ther.* 6, 1520–1535 (1999)
81. Okegawa, T. *et al.* The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. *Cancer Res.* 60, 5031–5036 (2000)
82. Li, Y. *et al.* Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. *Cancer Res.* 59, 325–330 (1999)
83. Bewley, M. C., Springer, K., Zhang, Y. B., Freimuth, P. & Flanagan, J. M. Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science* 286, 1579–1583 (1999).
84. Hoganson, D. K., Sosnowski, B. A., Pierce, G. F. & Doukas, J. Uptake of adenoviral vectors via fibroblast growth factor receptors involves intracellular pathways that differ from the targeting ligand. *Mol. Ther.* 3, 105–112 (2001).
85. Cripe, T. P. *et al.* Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus-adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. *Cancer Res.* 61, 2953–2960 (2001)
A good example of retargeting adenovirus to infect cells through non-CAR-mediated interactions. These approaches are designed to overcome the fact that many cancer cells downregulate CAR expression, whereas CAR is abundantly expressed in many normal epithelial cells.
86. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G. & Lieber, A. Efficient gene transfer into human CD34+ cells by a retargeted adenovirus vector. *J. Virol.* 74, 2567–2583 (2000).
87. Fisher, K. D. *et al.* Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther.* 8, 341–348 (2001).
88. Ikeda, K. *et al.* Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. *Nature Med.* 5, 881–887 (1999).
89. Chen, Y., Yu, D. C., Charlton, D. & Henderson, D. R. Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy. *Hum. Gene Ther.* 11, 1553–1567 (2000)
90. McCormick, F. New-age drug meets resistance. *Nature* 412, 281–282 (2001).
91. Grim, J. *et al.* Adenovirus-mediated delivery of p16 to p16-deficient human bladder cancer cells confers chemoresistance to cisplatin and paclitaxel. *Clin. Cancer Res.* 3, 2415–2423 (1997)
92. Patel, S. D. *et al.* The p53-independent tumoricidal activity of an adenoviral vector encoding a p27–p16 fusion tumor suppressor gene. *Mol. Ther.* 2, 161–169 (2000).
93. Riley, D. J., Nikitin, A. Y. & Lee, W. H. Adenovirus-mediated retinoblastoma gene therapy suppresses spontaneous pituitary melanotroph tumors in $Rb^{+/-}$ mice. *Nature Med.* 2, 1316–1321 (1996)
94. Claudio, P. P. *et al.* RB2/p130 gene-enhanced expression down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in vivo. *Cancer Res.* 61, 462–468 (2001).
95. Kawabe, S. *et al.* Adenovirus-mediated wild-type p53 gene expression radiosensitizes non-small cell lung cancer cells but not normal lung fibroblasts. *Int. J. Radiat. Biol.* 77, 185–194 (2001).
96. Nielsen, L. L. *et al.* Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. *Cancer Gene Ther.* 4, 129–138 (1997).
97. Kataoka, M. *et al.* An agent that increases tumor suppressor transgene product coupled with systemic transgene delivery inhibits growth of metastatic lung cancer *in vivo*. *Cancer Res.* 58, 4761–4765 (1998).
98. Spitz, F. R. *et al.* *In vivo* adenovirus-mediated p53 tumor suppressor gene therapy for colorectal cancer. *Anticancer Res.* 16, 3415–3422 (1996).
99. Czubayko, F. *et al.* Adenovirus-mediated transduction of ribozymes abrogates HER2/neu and pleiotrophin expression and inhibits tumor cell proliferation. *Gene Ther.* 4, 943–949 (1997)
100. Lui, V. W., He, Y. & Huang, L. Specific down-regulation of HER2/neu mediated by a chimeric U6 hammerhead ribozyme results in growth inhibition of human ovarian carcinoma. *Mol. Ther.* 3, 169–177 (2001)
101. Suzuki, T. *et al.* Adenovirus-mediated ribozyme targeting of HER2/neu inhibits *in vivo* growth of breast cancer cells. *Gene Ther.* 7, 241–248 (2000).
102. Tang, C. K. *et al.* Ribozyme-mediated down-regulation of ErbB-4 in estrogen receptor-positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo*. *Cancer Res.* 59, 5315–5322 (1999).
103. Aoki, K. *et al.* Growth inhibitory effect of anti-KRAS adenovirus on lung cancer cells. *Cancer Gene Ther.* 3, 295–301 (1996).
104. Tsuchida, T. *et al.* Adenovirus-mediated anti-KRAS ribozyme induces apoptosis and growth suppression of human pancreatic carcinoma. *Cancer Gene Ther.* 7, 373–383 (2000)
105. Funato, T., Ishii, T., Kambe, M., Scanlon, K. J. & Sasaki, T. Anti-KRAS ribozyme induces growth inhibition and increased chemosensitivity in human colon cancer cells. *Cancer Gene Ther.* 7, 495–500 (2000).
106. Zhang, Y. A., Nemunaitis, J., Scanlon, K. J. & Tong, A. W. Anti-tumorigenic effect of a KRAS ribozyme against human lung cancer cell line heterotransplants in nude mice. *Gene Ther.* 7, 2041–2050 (2000)
107. Irie, A. *et al.* Therapeutic efficacy of an adenovirus-mediated anti-HRAS ribozyme in experimental bladder cancer. *Antisense Nucleic Acid Drug Dev.* 9, 341–349 (1999).
108. Kunke, D. *et al.* Preclinical study on gene therapy of cervical carcinoma using adeno-associated virus vectors. *Cancer Gene Ther.* 7, 766–777 (2000)
109. Potter, P. M. *et al.* Construction of adenovirus for high level expression of small RNAs in mammalian cells. Application to a BCL2 ribozyme. *Mol. Biotechnol.* 15, 105–114 (2000)
110. Ludwig, A. *et al.* Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. *Cancer Res.* 61, 3053–3061 (2001)

111. Jiang, W. G. *et al.* A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-MET and reduces migration and invasiveness of breast cancer cells. *Clin. Cancer Res.* 7, 2555–2562 (2001).
112. Cheng, J. *et al.* Inhibition of cell proliferation in HCC-9204 hepatoma cells by a c-MYC specific ribozyme. *Cancer Gene Ther.* 7, 407–412 (2000).
113. Trinh, Q. T., Austin, E. A., Murray, D. M., Knick, V. C. & Huber, B. E. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. *Cancer Res.* 55, 4808–4812 (1995).
114. Hanna, N. N. *et al.* Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. *Cancer Res.* 57, 4205–4209 (1997).
115. Bridgewater, J. A. *et al.* Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur. J. Cancer* 31A, 2362–2370 (1995).
116. Danks, M. K. *et al.* Overexpression of a rabbit liver carboxylesterase sensitizes human tumour cells to CPT-11. *Cancer Res.* 58, 20–22 (1998).
117. Waxman, D. J. *et al.* Cytochrome P450-based cancer gene therapy: recent advances and future prospects. *Drug Metab. Rev.* 31, 503–522 (1999).
118. Sorscher, E. J. *et al.* Tumor cell bystander killing in colonic carcinoma utilizing the *Escherichia coli DeoD* gene to generate toxic purines. *Gene Ther.* 1, 233–238 (1994).
119. Topf, N., Worgall, S., Hackett, N. R. & Crystal, R. G. Regional 'pro-drug' gene therapy: intravenous administration of an adenoviral vector expressing the *E. coli* cytosine deaminase gene and systemic administration of 5-fluorocytosine suppresses growth of hepatic metastasis of colon carcinoma. *Gene Ther.* 5, 507–513 (1998).
120. Gnant, M. F., Puhlmann, M., Alexander, H. R., Jr & Bartlett, D. L. Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. *Cancer Res.* 59, 3396–3403 (1999).
121. Block, A. *et al.* Gene therapy of metastatic colon carcinoma: regression of multiple hepatic metastases by adenoviral expression of bacterial cytosine deaminase. *Cancer Gene Ther.* 7, 438–445 (2000).
122. Pandha, H. S. *et al.* Genetic prodrug activation therapy for breast cancer: a phase I clinical trial of ERBB2-directed suicide gene expression. *J. Clin. Oncol.* 17, 2180–2189 (1999).
123. Suzuki, S., Tadakuma, T., Asano, T. & Hayakawa, M. Coexpression of the partial androgen receptor enhances the efficacy of prostate-specific antigen promoter-driven suicide gene therapy for prostate cancer cells at low testosterone concentrations. *Cancer Res.* 61, 1276–1279 (2001).
124. Miyauchi, M. *et al.* Expression of herpes simplex virus-thymidine kinase gene controlled by a promoter region of the midkine gene confers selective cytotoxicity to ganciclovir in human carcinoma cells. *Int. J. Cancer* 91, 723–727 (2001).
125. Ido, A. *et al.* Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human α-fetoprotein promoter. *Cancer Res.* 61, 3016–3021 (2001).
126. Morimoto, E., Inase, N., Miyake, S. & Yoshizawa, Y. Adenovirus-mediated suicide gene transfer to small cell lung carcinoma using a tumor-specific promoter. *Anticancer Res.* 21, 329–331 (2001).
127. You, L., Yang, C. T. & Jablons, D. M. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res.* 60, 1009–1013 (2000).
128. Xu, X. *et al.* Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. *Hum. Gene Ther.* 12, 549–561 (2001).
129. Koeneman, K. S. *et al.* Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J. Urol.* 18, 102–110 (2000).
130. Chen, R. H. & McCormick, F. Selective targeting to the hyperactive β-catenin/T-cell factor pathway in colon cancer cells. *Cancer Res.* 61, 4445–4449 (2001).
131. Hernandez-Alcoceba, R., Pihalja, M., Wicha, M. S. & Clarke, M. F. A novel, conditionally replicative adenovirus for the treatment of breast cancer that allows controlled replication of E1a-deleted adenoviral vectors. *Hum. Gene Ther.* 11, 2009–2024 (2000).
132. Majumdar, A. S. *et al.* The telomerase reverse transcriptase promoter drives efficacious tumor suicide gene therapy while preventing hepatotoxicity encountered with constitutive promoters. *Gene Ther.* 8, 568–578 (2001).
133. Zhang, R., Straus, F. H. & DeGroot, L. J. Adenoviral-mediated gene therapy for thyroid carcinoma using thymidine kinase controlled by thyroglobulin promoter demonstrates high specificity and low toxicity. *Thyroid* 11, 115–123 (2001).
134. Nishino, K. *et al.* Adenovirus-mediated gene therapy specific for small cell lung cancer cells using a Myc–Max binding motif. *Int. J. Cancer* 91, 851–856 (2001).
135. Ueda, K. *et al.* Enhanced selective gene expression by adenovirus vector using Cre/*loxP* regulation system for human carcinoembryonic antigen-producing carcinoma. *Oncology* 59, 255–265 (2000).
136. Lan, K. H. *et al.* Tumor-specific gene expression in carcinoembryonic antigen-producing gastric cancer cells using adenovirus vectors. *Gastroenterology* 111, 1241–1251 (1996).
137. Siders, W. M., Halloran, P. J. & Fenton, R. G. Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res.* 56, 5638–5646 (1996).
138. Chen, J. *et al.* A glial-specific, repressible, adenovirus vector for brain tumor gene therapy. *Cancer Res.* 58, 3504–3507 (1998).
139. Walton, T. *et al.* Endothelium-specific expression of an E-selectin promoter recombinant adenoviral vector. *Anticancer Res.* 18, 1357–1360 (1998).
140. Manome, Y. *et al.* Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the Egr-1 promoter: activation by ionizing radiation or uptake of radioactive iododeoxyuridine. *Hum. Gene Ther.* 9, 1409–1417 (1998).
141. Gotoh, A. *et al.* Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J. Urol.* 160, 220–229 (1998).
142. McKie, E. A., Graham, D. I. & Brown, S. M. Selective astrocytic transgene expression *in vitro* and *in vivo* from the GFAP promoter in a HSV RL1 null mutant vector — potential glioblastoma targeting. *Gene Ther.* 5, 440–450 (1998).
143. Parr, M. J. *et al.* Tumor-selective transgene expression *in vivo* mediated by an E2F-responsive adenoviral vector. *Nature Med.* 3, 1145–1149 (1997).
144. Miyao, Y. *et al.* Usefulness of a mouse myelin basic protein promoter for gene therapy of malignant glioma: myelin basic protein promoter is strongly active in human malignant glioma cells. *Jpn J. Cancer Res.* 88, 678–686 (1997).
145. Ozaki, K. *et al.* Use of von Willebrand factor promoter to transduce suicidal gene to human endothelial cells, HUVEC. *Hum. Gene Ther.* 7, 1483–1490 (1996).
146. Anderson, L. M., Krotz, S., Weitzman, S. A. & Thimmapaya, B. Breast cancer-specific expression of the *Candida albicans* cytosine deaminase gene using a transcriptional targeting approach. *Cancer Gene Ther.* 7, 845–852 (2000).

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ABL | *APC* | BCR | *BRCA1* | *BRCA2* | CAR | β-catenin | cyclin D1 | cytochrome P450 | E2F | EGFR | endostatin | ERBB2 | fibroblast growth-factor receptor | *HRAS* | IGF-1 | IGF1BP | *INK4A* | matrilysin | MDM2 | MUC1 | c-MYC | p300 | probasin | PSA | *PTEN* | *RB* | TCF-4 | thrombospondin | TIMP-2 | *TP53* | tumour necrosis factor | VEGF

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structure of CAR

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Exhibit B

Oncogene (2001) 20, 7855–7865

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The role of adenovirus E4orf4 protein in viral replication and cell killing

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It has only been within the last few years that insights have been gained into the remarkable diversity of functions of the adenovirus early transcription region 4 (E4) products. The polypeptide encoded by E4 open reading frame 4 (E4orf4) has emerged as an enigmatic product. Although it accomplishes certain functions that propel viral replication, it has also been shown to be highly toxic, an effect that could dampen the infectious cycle, but that also might serve to facilitate release of viral progeny. When expressed alone, E4orf4 induces a novel form of p53-independent apoptosis in cancer cells but not in normal human cells, thus making it of potential use in cancer gene therapy. In addition, knowledge of its mechanism of action, especially with regard to its interaction with protein phosphatase 2A (PP2A), could provide insights to develop new small molecule anti-cancer drugs. Thus future studies on E4orf4 should be both informative and potentially valuable therapeutically. In this study we review the current status of knowledge on E4orf4. *Oncogene* (2001) **20**, 7855–7865.

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Early region 4 (E4)

The E4 transcription unit contains at least seven open reading frames (Freyer *et al.*, 1984; Herisse *et al.*, 1981; Virtanen *et al.*, 1984) that, until recently, appeared to encode functions with no obvious common theme. It is now clear that most, if not all, yield products that play important roles in furthering both early and late stages of the adenovirus infectious cycle (for review see Täuber and Dobner, this issue). Some, including E4orf1, E4orf3 and E4orf6, also can induce or enhance cell transformation either alone or in cooperation with products of early region 1A and 1B (E1A and E1B).

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E4orf4 structure and synthesis

The product of E4 open reading frame 4 (E4orf4; also reviewed in Kleinberger, 2000) is a small polypeptide, in the case of Ad2 or Ad5, consisting of 114 residues (see Figure 1). It shares no extensive sequence homology with any known protein. E4orf4 contains a highly basic sequence (in Ad2, R^{86}AKRRDRRRR; consensus RxKRRxRRRR); however, it is still unclear if this sequence serves as a nuclear targeting or retention signal. It also contains a proline-rich sequence near the amino terminus (in Ad2, M^{1}VLPALPAPP; consensus MxxPxLPxPP) that could potentially represent an SH3-binding site. Apart from small regions at the amino and carboxy termini, deletion of even short portions of E4orf4 yields products that are generally non-functional and often somewhat unstable (Marcellus *et al.*, 2000). These results suggest that the central core of the E4orf4 protein may contain considerable tertiary structure of functional importance. E4orf4 mRNA is produced from the E4 promoter early after infection as one of seven E4 transcripts; however, transcription of E4 ceases during the late phase. Nevertheless, E4orf4 persists at constant levels even late in infection as it is highly stable (Boivin *et al.*, 1999). Studies on post-translational modifications of E4orf4 have not been carefully pursued, but low levels of both phosphoserine and phosphothreonine have been detected in E4orf4 isolated from infected cells (J Lee and PE Branton, unpublished).

Early studies of E4orf4 function using mutants

Early studies that employed mutants defective in individual E4 products indicated that E4orf4 (and most other E4 products) is not essential for virus growth (Halbert *et al.*, 1985). E4orf4 function was linked to viral DNA synthesis in studies showing that viruses lacking E4orf4 replicated viral DNA efficiently, but that a mutant expressing E4orf4 as the only E4 product was highly defective (Bridge *et al.*, 1993). Whether these effects are due to the toxicity of E4orf4, discussed below, and/or to the essential role of other E4 products in DNA synthesis is not clear.

Studies in S49 mouse lymphoma cells showed that expression of the adenovirus E1A protein and addition of dibutyryl cAMP induces transcription factor AP-1

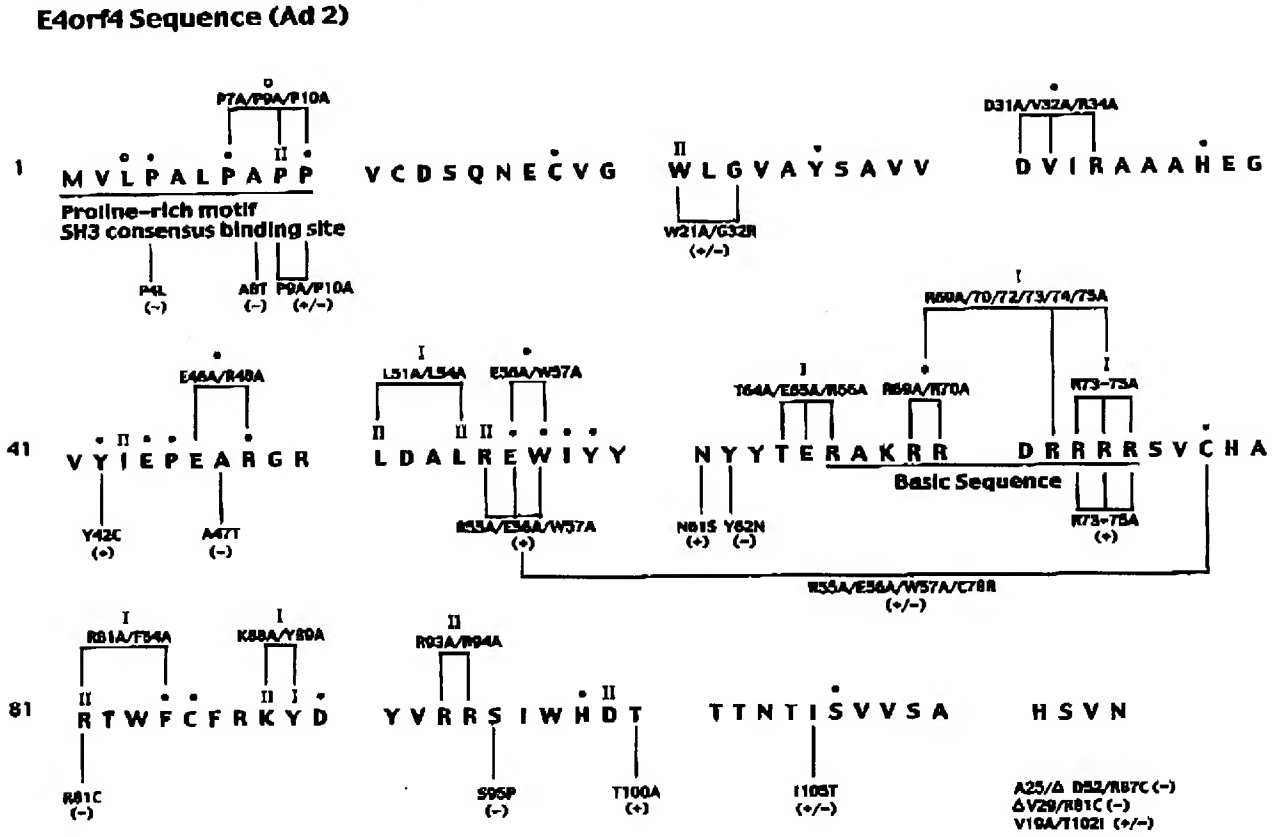

Figure 1 E4orf4 sequence and mutant information. The single amino acid sequence of Ad2 E4orf4 is shown. The positions of proline-rich and basic regions are underlined. Above are indicated positions of point mutations. The ability of these mutants to induce cell death and interact with PP2A are correlated and grouped as class I, class II, wild-type ● or unstable ○ (Marcellus *et al.*, 2000). Below are point mutants as determined from separate studies and their ability to promote apoptosis in transformed cell lines. (+) = induces apoptosis at wild type levels; (−) = no apoptosis; (+/−) = induces intermediate level of apoptosis (Shtrichman *et al.*, 1999; Afifi *et al.*, 2001)

activity through increased expression of genes encoding its constituents, JunB and c-Fos, and increased translation of *c-Fos* mRNA (Engel *et al.*, 1988; Müller *et al.*, 1989). At fairly early times during infection all such activities are reduced, and this reduction was shown to be due to the E4orf4 protein (Müller *et al.*, 1989). E4orf4 also negatively regulates expression of the E4 promoter (Bondesson *et al.*, 1996), and more recently it has been found to reduce expression of the viral E2 promoter regulated by transcription factor E2F (Mannervik *et al.*, 1999). Thus E4orf4 elicits a strong dampening effect on E1A-activated transcription. E4orf4 also plays a key role in controlling the splicing pattern of late adenovirus L1 mRNAs (Kanopka *et al.*, 1998).

E4orf4 interacts with protein phosphatase 2A (PP2A)

The major first insight into the mechanism of action of E4orf4 came with the observation that immunoprecipitates prepared using anti-E4orf4 antibodies also contain the Bα regulatory subunit as well as the A and C subunits of the serine/threonine phosphatase PP2A (Kleinberger and Shenk, 1993). It is likely, though not proven biochemically, that a direct interaction takes place between E4orf4 and Bα. This finding suggested a hypothesis, which persists to the present, that the biological effects of E4orf4 are induced by a change in PP2A activity, substrate specificity, or localization following interaction with the B subunit of the holoenzyme.

Results from several early studies support the idea that changes in phosphorylation underlie E4orf4 function. Downregulation of AP-1 activity by E4orf4 is associated with hypophosphorylation of both c-Fos and the E1A protein (Müller *et al.*, 1992). Phosphorylation of c-Fos regulates its activation or repression functions as a component of the heterodimeric transcription factor AP-1. Thus the downregulation of transcription of the *junB* and c-*fos* genes, and thus

AP-1 activity, could result from underphosphorylation of c-Fos, either directly or indirectly by E4orf4-regulated PP2A. The time required for this effect on AP-1 suggested that PP2A may play an indirect role, possibly through downregulation of protein kinase(s) that act on c-Fos sites (Kleinberger and Shenk, 1993; Müller *et al.*, 1992). Nonetheless, treatment of cells with okadaic acid, a relatively specific inhibitor of PP2A, blocks the ability of E4orf4 to induce c-Fos hypophosphorylation (Kleinberger and Shenk, 1993). The basis for E4orf4-induced decreases in c-Fos translation are not known, but perhaps suggest that E4orf4 regulates protein synthesis.

Although the mechanism of E4orf4-mediated effects on E2F are not understood (Mannervik *et al.*, 1999), some insights have been gained on regulation of E4 expression by E4orf4. It is believed that at least two phosphorylation events control this process. E4 promoter expression is regulated by ATF2 and more stringently by transcription factor E4F, which binds to two specialized ATF2 sites. E4orf4 downregulation of E4 expression was found to be inhibited by okadaic acid, suggesting that the interaction between E4orf4 and PP2A may be involved (Bondesson *et al.*, 1996). It is presumed therefore that changes in phosphorylation of a transcription factor critical for the E4 promoter is involved. In this regard, E4F is known to be regulated by E1A through phosphorylation (Fernandes and Rooney, 1997), and thus could represent a key E4orf4-PP2A target. E4 transcription also absolutely requires E1A proteins, specifically the CR3 transactivation region and auxiliary region AR1 (Bondesson *et al.*, 1992). E4orf4 induces a loss in phosphorylation of E1A protein (Müller *et al.*, 1992), and these sites were mapped to serine residues 185 and 188, which lie within the CR3-AR1 region (Whalen *et al.*, 1997). Further, this study showed that hyperphosphorylation of these E1A sites through expression of constitutively active MAPKK increased transcription from the E4 promoter but not from that of E3. Although another study suggested a reduced importance of E1A phosphorylation (Bondesson *et al.*, 1996), these results suggested that hypophosphorylation of these sites in E1A protein could play a key role in E4orf4/PP2A-induced downregulation of E4 expression. As was the case with c-Fos, it is unclear if E4orf4-regulated PP2A dephosphorylates E1A protein or if the effect is indirect and via effects on the E1A kinase, believed to be a MAPK-like enzyme (Whalen *et al.*, 1997). It is tempting to postulate that downregulation of E4 expression serves to maintain low levels of E4orf4, which is highly toxic (see below).

A final link between PP2A and E4orf4 function concerns regulation of L1 mRNA splicing late in infection. E4orf4 enhances usage of the distal L1 acceptor site to induce the accumulation of IIIa mRNA at the expense of the 52,55K mRNA formed via the proximal site (Kanopka *et al.*, 1998). SR splicing factors inhibit IIIa pre-mRNA splicing by binding to the intronic IIIa repressor element (Kanopka *et al.*, 1996). During adenovirus infection some SR factors become functionally inactivated as IIIa repressors through virus-induced dephosphorylation, thus enhancing L1-IIIa mRNA formation (Kanopka *et al.*, 1998). It has recently been shown that E4orf binds, either directly or indirectly, to a subset of hyperphosphorylated SR proteins, and that E4orf4 mutants that fail to interact with the B subunit of PP2A fail to activate IIIa splicing (Estmer-Nilsson *et al.*, 2001). These results suggest that E4orf4-associated PP2A may be responsible for dephosphorylating these SR factors to promote a switch in the late adenovirus splicing pattern. Thus effects of E4orf4 on both transcription and splicing could be mediated through dephosphorylation by E4orf4-regulated PP2A.

A curious result is the apparent inhibition by E4orf4 of CTL lysis of cells infected by adenovirus vectors and harboring E4orf4 in the vector backbone (Kaplan *et al.*, 1999). This response was unaffected by treatment with okadaic acid, suggesting that PP2A is not involved.

Structure and function of PP2A

PP2A is an abundant serine/threonine phosphatase. Figure 2 shows that its holoenzyme is a trimer of catalytic C subunit, and A and B regulatory subunits (Janssens and Goris, 2001). The A subunit is a 65 kDa rod-like protein containing 15 non-identical repeats. The 37 kDa catalytic C subunit binds to repeats 11–15, whereas B subunits bind to repeats 1–10. A and C are ubiquitous, and each exists in two isoforms. Thus far about 20 B subunit variants have been cloned and exist in three classes. The B (B55) class comprises four

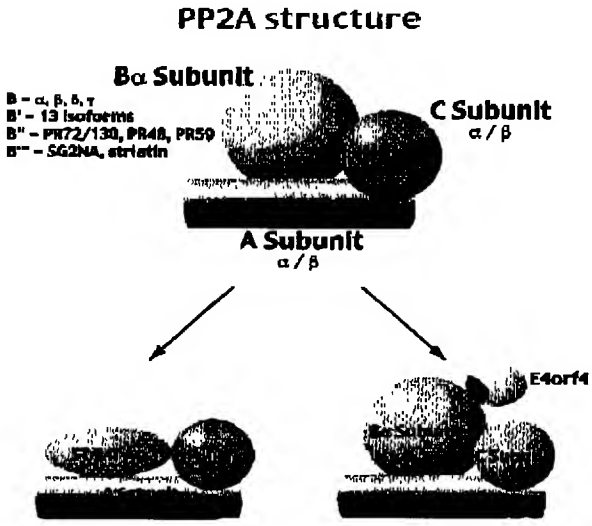

Figure 2 Structure of the PP2A holoenzyme. The A, B and C subunits of PP2A have been presented. The figure shows the effects of interaction with E4orf4 and ST antigens

members of about 55 kD: Bα, Bβ, Bγ and Bδ (Healy *et al.*, 1991; Khew-Goodall *et al.*, 1991; Pallas *et al.*, 1992; Zolnierowicz *et al.*, 1994). The B′ (B56) class (α, β, γ, δ, ε) is composed of at least 13 isoforms (Csortos *et al.*, 1996; McCright *et al.*, 1996a; McCright and Virshup, 1995; Tanabe *et al.*, 1996; Tehrani *et al.*, 1996; Zhao *et al.*, 1997; Zolnierowicz *et al.*, 1996). The B″ class contains at least three isoforms PR72/130, PR48 and PR59 (Hendrix *et al.*, 1993; Voorhoeve *et al.*, 1999; Yan *et al.*, 2000). Recently, two additional B-type subunits, S/G_2 nuclear autoantigen and striatin, have been identified and represent a putative B‴ (B93/110) family (Moreno *et al.*, 2000). Different classes of B subunits share little or no homology, yet all bind to a similar region of the A subunit. Recent evidence has shown that binding of B subunits to the AC core is regulated by both phosphorylation and methylation of the C subunit (Evans and Hemmings, 2000; Wei *et al.*, 2001; Wu *et al.*, 2000). B subunits can be located either in the cytoplasm or nucleus. Some, like Bα, are expressed in many tissues, while others have more limited expression. B subunits function not only in defining the substrate specificity of PP2A but also in intracellular targeting, tissue specificity, and as binding partners for interacting proteins. PP2A substrates include proteins involved in basal metabolism, DNA replication, cell proliferation, and cell cycle regulation (Janssens and Goris, 2001). The AC (core) dimer is also present at reasonable levels but lacks substrate specificity (Kremmer *et al.*, 1997). A number of important PP2A substrates involved in growth regulation have been identified, such as cyclin-dependent kinases (Cdks) and their substrates, including possibly the retinoblastoma tumor suppressor (Agostinis *et al.*, 1992; Ferrigno *et al.*, 1993). PP2A downregulates the MAPK pathway by dephosphorylating MAPK and MAPKK (Frost *et al.*, 1994; Karin and Hunter, 1995; Sontag *et al.*, 1993). There is also mounting evidence that both the Aα and Aβ subunits may function as tumor suppressors as they are frequently mutated in melanomas, lung and breast carcinomas and gliomas (Calin *et al.*, 2000; Wang *et al.*, 1998; Colella *et al.*, 2001).

Interaction of small DNA tumor virus tumor antigens with PP2A

In addition to adenovirus E4orf4, tumor antigens encoded by other small DNA tumor viruses also bind to PP2A and these interactions may be of significance in transformation or immortalization by these agents. Small T antigens (ST) of SV40 and polyoma and the polyoma middle T antigen (MT) bind PP2A to form stable ST/MT-A-C heterotrimers with the release of free B subunit (Pallas *et al.*, 1990; Walter *et al.*, 1990). Interactions of PP2A with ST enhance cell survival, and with MT they are required for binding of c-Src, PI3′ kinase and Shc, functions that are essential for oncogenesis (Campbell *et al.*, 1995; Glenn and Eckhart, 1995). T antigens bind to the A subunit at repeats 3–6 (SV40-ST) or 2–8 (polyoma ST/MT) and, as these sites overlap those utilized by the B subunit, these interactions displace B. Binding destroys the holoenzyme and inhibits overall core enzyme activity; however, this PP2A complex appears to act on SV40 ST antigen and p53 (Scheidtmann *et al.*, 1991; Yang *et al.*, 1991). It is generally assumed that the biological effects of these interactions are to modify or inhibit PP2A activity; however, it remains possible that released free B subunits carry out an as yet unidentified function. It is still unclear whether or not T antigen-modified PP2A retains activity against some cellular substrates. One possibility is that global inhibition of PP2A by SV40 and polyoma tumor antigens prevents activation by PP2A of pro-apoptotic cellular factors, thus enhancing cell survival and transformation. It is also possible that the modified complex is redirected to substrates which, when dephosphorylated, promote survival. As mentioned above, E4orf4 may activate PP2A, and as discussed in detail below, one consequence is to induce cell death by apoptosis.

Features of apoptosis

Apoptosis is a genetically-regulated killing process characterized by shrinkage and rounding of cells, disruption of the cytoskeleton, condensation of chromatin, cleavage of DNA to nucleosome sized fragments, cytoplasmic vacuolization and blebbing, and in the final stages, fragmentation of the cell into membrane-bound apoptotic bodies that are engulfed by neighboring cells. In this way cells containing genotoxic damage, imbalances in growth (such as cancer cells), or those challenged by virus infection are eliminated from the host. Apoptosis is often regulated at two checkpoints (Gross *et al.*, 1999; Korsmeyer, 1999). Common to most (but not all) apoptotic pathways is the activation of caspases (Salvesen and Dixit, 1999). Caspases represent a unique class of pro-proteases that can be activated directly, as with TNFR and Fas, which, after ligand binding, form signaling complexes that bind and activate initiator caspases (Nagata, 1997). With many apoptosis inducers, including p53, activation depends on a signal from an upstream checkpoint regulated by dimerization of a family of integral membrane proteins related to Bcl-2. Homodimerization of Bcl-2 family member Bax promotes caspase activation that is prevented by Bcl-2 and related proteins that heterodimerize with Bax to prevent formation of Bax-Bax homodimers. Another Bcl-2-related protein, Bad, triggers apoptosis by binding Bcl-2, thus preventing heterodimerization with Bax. Cells are maintained by 'survival factors' and the positive balance of death suppressors. Dimerization occurs via Bcl-2 homology (BH) domains. Bcl-2 family members are components of membrane complexes in mitochondria and the endoplasmic reticulum and may constitute channels that regulate release of initiators of caspases, like cytochrome *c*. Activation of the caspase cascade results in cleavage and activation of enzymes that kill the cell.

Signaling and phosphorylation in the apoptotic pathway

Many 'survival factors' are growth factors that regulate cell proliferation through phosphorylation involving tyrosine kinase receptors and serine/threonine kinase cascades. Bcl-2 and Bcl-X_L are both positively and negatively regulated by serine phosphorylation (Yamamoto *et al.*, 1999). Phosphorylation of pro-apoptotic Bad blocks binding to Bcl-2 and promotes association with 14-3-3 protein in the cytoplasm (Zha *et al.*, 1996). PI3' kinase is linked with phosphorylation of Bad. PI3' kinase is activated by binding to phosphotyrosylated receptors and resulting phospholnositides recruit the kinases Akt and PDK1 via PH domains (Hemmings, 1997a,b). PDK1 activates Akt by phosphorylation and Akt subsequently translocates and inactivates Bad by phosphorylating some sites (Datta *et al.*, 1997; del Peso *et al.*, 1997). Thus phosphorylation of Bcl-2 and Bad can promote cell survival. Therefore dephosphorylation of these proteins by PP2A or other phosphatases could promote apoptosis.

Induction and suppression of apoptosis by adenovirus products

Infection of cells by many viruses induces apoptosis (Roulston *et al.*, 1999; Teodoro and Branton, 1997). In the case of adenoviruses, the principal cause for this induction is the E1A protein (see Figure 3). A major role therefore of the E1B55K, E1B19K and E4orf6 products is to block E1A toxicity long enough to allow high virus yields. This effect is also evident in transformation of rodent cells, as expression of E1A alone results in rare transformants that normally die from apoptosis. The best understood mechanism of E1A-induced apoptosis relates to enhanced stabilization and activation of p53. Such stabilization results from interactions of E1A proteins with either the p300/CBP histone acetyl transferases or the RB tumor suppressor family (Chiou and White, 1997; Querido *et al.*, 1997). The mechanism of the latter is related to enhanced expression of Arf by E2F-1 and thus inhibition of Mdm2-mediated p53 degradation and induction of p53-dependent apoptosis (de Stanchina *et al.*, 1998). Viability of infected or transformed cells in the presence of E1A is enhanced by the E1B55K protein, which binds to and blocks p53 function; by the E1B19K protein, which functions as a Bcl-2 analog to suppress apoptosis; by the E4orf6 protein, which binds to p53 and inhibits p53 transactivation activity; and by E4orf6/E1B55K complexes, which target p53 for degradation via a Cul5-mediated ubiquitination pathway (see review by Täuber and Dobner in this issue).

Induction of apoptosis by E4orf4

Because E1A products appeared to induce apoptosis largely via p53, our group reasoned that expression of E1A in p53-null cells should not result in apoptotic cell death. It was found that such was the case in p53 'knockout' mouse cells infected with adenoviruses expressing only a CR3-defective 243R E1A product. Due to the absence of the CR3 transactivation region, this virus expresses E1A and E1B products, low levels of E2 proteins, but almost no E3 or E4 products. Surprisingly, a similar experiment conducted with viruses expressing full length E1A induced apoptosis in p53-null cells, as evidenced by the generation of DNA ladders and chromatin condensation characteristic of apoptosis (Teodoro *et al.*, 1995). These results suggested that apoptosis was caused not by E1A but rather by another viral or cellular product expressed under the control of the E1A transactivation region. To identify this product studies were conducted in E1A-expressing p53-null mouse cells infected with adenovirus vectors lacking various portions of the viral genome, and the results indicated that one or more E4 proteins were responsible (Marcellus *et al.*, 1996). Using a series of E4 mutants, the killing function was mapped to E4orf4 (Marcellus *et al.*, 1998). With virus in which E4orf4 or E4orf4 plus either E4orf6 or E4orf3 were the only E4 products, cell death was induced at high efficiency. Expression of E4orf4 alone in the absence of all other Ad products also induced p53-independent cell death by apoptosis (Marcellus *et al.*, 1998). Similar effects were observed with E4orf4 products from all classes of human adenoviruses (Marcellus *et al.*, 2000). We also established an E4orf4-inducible CHO cell line and showed that such cells die upon induction of E4orf4, exhibiting classic apoptotic features including DNA degradation, chromatin condensation, and the presence of phosphatidyl serine on the outer cell membrane as determined by annexin V staining (Lavoie *et al.*, 1998). Curiously, apoptosis was not affected by the pan-caspase inhibitor

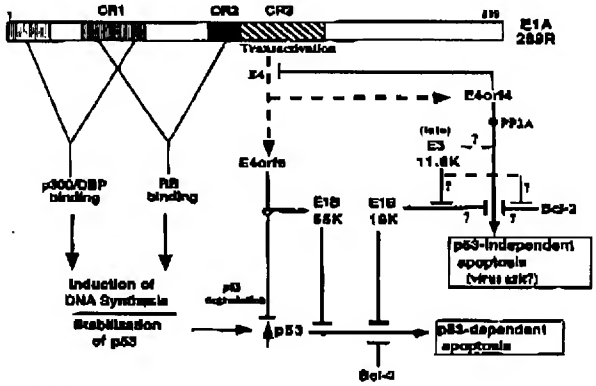

Figure 3 Induction and suppression of apoptosis by human adenoviruses. The Ad5 E1A protein (containing conserved regions CR1, CR2 and CR3) is shown at the top. Solid lines indicate biological effects, either positive (arrows) or negative (bars). Dotted lines indicate transactivation of E4 transcription by E1A protein. The arrow beside p53 indicates a rise in p53 levels. The O's represent complex formation (E1B55K/E4orf6 and E4orf4/PP2A)

zVAD-fmk, and thus did not appear to involve significant activation of caspases (see more below). As this work was being carried out, the group of Tamar Kleinberger was continuing studies on E4orf4 and they showed independently that E4orf4 induces p53-independent apoptosis (Shtrichman and Kleinberger, 1998).

The finding that E4orf4 is a toxic protein was originally surprising, as earlier work showed that a viral mutant lacking E4orf4 produced an early cytotoxic effect characterized by the release of cells from the monolayer (Müller *et al.*, 1992). In retrospect, this result is difficult to interpret because, as discussed above, E4orf4-null viruses are defective in several aspects of the lytic cycle and overexpress other E4 products. Thus such effects could alter cell physiology and lead to reduced attachment to the plate. Analysis of these cells showed that they were not dead, as indicated by Trypan Blue exclusion, and in fact E4orf4-null viruses exhibit a prolonged delay in cell killing (Marcellus *et al.*, 1998). These results suggested a hypothesis, which remains unproven, that E4orf4 plays a role in the death of infected cells by apoptosis. Death of infected cells by apoptosis could afford several survival advantages, and this mechanism may be common to many viruses (Roulston *et al.*, 1999; Teodoro and Branton, 1997). The inflammatory response would be reduced because apoptotic cells are compartmentalized into lipid vesicles that are taken up rapidly by neighboring cells. This effect would not only lessen the immune response, but also facilitate receptor-independent spread of the virus. Such progeny viruses would also be protected from host antibodies and proteases. As discussed below, E4orf4 killing appears to be selective for cancer cells; however, E4orf4 has evolved in the presence of the E1A oncogene, which may establish suitable conditions for induction of apoptosis in lytically-infected cells. If E4orf4 killing plays a role in virus release, why do adenovirus-infected cells not die earlier, as E4orf4 is expressed at early times? First, even when expressed alone at much higher levels than in infected cells, E4orf4 takes 24–48 h to cause significant cell death. Second, the Bcl-2-like E1B19K protein may delay cell death, as both Bcl-2 and Bcl-X_L have been suggested to reduce E4orf4-induced cell killing (Lavoie *et al.*, 1998). And third, although E4orf4 is highly toxic, it is not sufficient for the efficient killing of infected cells. Studies on adenovirus mutants lacking the E3-11.6K protein, which is expressed very late in infection, demonstrated a prolonged delay in cell killing even in the presence of E4orf4. Further experiments are required to understand the mechanism of adenovirus-induced death of infected cells and the potential cooperation between E4orf4 and E3-11.6K.

Binding to PP2A is essential for cell killing by E4orf4

As discussed above, E4orf4 binds to the Bα subunit of PP2A and some of its biological effects appear to involve PP2A. To determine the role of this interaction in E4orf4 killing, two groups have conducted genetic studies in which the ability of E4orf4 mutant proteins to bind the Bα subunit and to induce cell killing were compared. Genetic analysis of E4orf4 is difficult as deletion of even short E4orf4 coding sequences yields largely non-functional and often unstable products (Marcellus *et al.*, 2000). Thus both groups generated a series of point mutations in the E4orf4 coding sequence, both randomly, and by selective mutagenesis affecting residues that are highly conserved in all adenovirus serotypes (Shtrichman *et al.*, 1999; Marcellus *et al.*, 2000). Both studies indicated a high degree of correlation between binding to PP2A and induction of cell death. That is, mutant E4orf4 proteins that fail to associate with C subunit or with high levels of PP2A phosphatase actiity or that bind very poorly or not at all to the Bα subunit are highly defective for killing. One of these studies, which employed a much larger array of mutants, found that two mutant classes were apparent: class I mutants (mapping largely between residues 51–89) that fail to bind Bα and fail to kill; and class II mutants that interact with Bα at fairly normal levels but are defective for killing (Marcellus *et al.*, 2000). Further support for the requirement of the Bα subunit was obtained in studies showing that expression of antisense RNA specific for Bα transcripts appeared to inhibit E4orf4 cell killing (Shtrichman *et al.*, 1999). These results indicate that binding to the Bα subunit is absolutely required for E4orf4-mediated cell death and thus that PP2A is the major target. The existence of class II mutants could suggest that Bα binding may not be sufficient and that a second E4orf4 function exists; however, it is possible that although binding takes place, it is non-functional in terms of eliciting appropriate effects on PP2A.

Specificity of B subunit binding

Several kinds of studies have indicated that association of E4orf4 with PP2A is via an interaction with the B subunit. Although binding to the Bα subunit of PP2A has been well established and found to take place in the yeast two hybrid system (Z Zhang, RC Marcellus and PE Branton, unpublished), it has not yet been shown rigorously whether this interaction is direct or via an intermediary protein. Studies with recombinant proteins should resolve this issue. As multiple B subunits exist in four families that share little sequence homology, it was of interest to determine the specificity of the E4orf4 interaction. In one study conducted in E1A/E1B-transformed human 293 cells, E4orf4 binding was also detected with members of the B′(B56) family of subunits (Shtrichman *et al.*, 2000). Although mutant analysis indicated that such binding was not involved in E4orf4-mediated cell killing, these observations raise the possibility that E4orf4 elicits other functions via PP2A holoenzymes containing B56 subunits. Our group has also analysed B subunit binding, using H1299 human lung carcinoma cells. Although high

levels of binding of E4orf4 were detected with all four highly related members of the B(B55) family (i.e. Bα, Bβ, Bγ and Bδ), none was detected with any members of the B'(B56), B'' or B''' subunit families, which are largely unrelated to B55 proteins (Marcellus *et al.*, submitted). It is possible that the failure to detect binding with B56 subunits is related to differences in antibodies or positioning of epitope tags on E4orf4 or B56 products used in binding analyses; however, after extensive efforts, such does not appear to be the case. It is also possible that B56 binding is affected by factors that differ between 293 and H1299 cells. Thus this issue remains unresolved.

Analysis of the apoptotic pathway induced by E4orf4

As mentioned earlier, E4orf4 induces most of the classic hallmarks of apoptosis; however, important differences from most other inducers exist. Treatment of CHO cells expressing E4orf4 under an inducible promoter with caspase inhibitor zVAD-fmk had little effect on cell death following induction of E4orf4 expression (Lavoie *et al.*, 1998). Further, no induction of caspase-3 or PARP cleavage was apparent in these cells, suggesting that E4orf4-induced apoptosis is caspase independent. More recently, this question has been re-addressed using cells transfected with plasmid DNA expressing E4orf4 (Livne *et al.*, 2001). In p53-null human H1299 cancer cells, some induction of caspase-3 activity was detected as was release of cytochrome *c* from mitochondria, a hallmark of caspase activation. In E1A/E1B-expressing human 293 cells, E4orf4-induced apoptosis, as measured by DAPI staining of aberrant nuclei, was diminished by overexpression of a caspase-8 dominant-negative mutant, an effect not seen with a similar caspase-9 mutant. These results suggest that E4orf4 could function through a caspase-8 pathway similar to death receptors. Our group has been addressing this question using a different approach, with apparently different findings (Szymborski and PE Branton, in preparation). Cells were infected with adenovirus vectors expressing inducible E4orf4 or the pro-apoptotic proteins p53 or tBID. Caspase activation was assessed by Western blotting to detect the presence of cleaved, activated caspases. In H1299 cells, whereas p53 induced massive activation of caspases, expression of E4orf4 failed to activate caspases 1, 2, 3, 6, 7, 8 or 9 in dying cells. Similar results were also obtained in 293 cells using tBID as a positive control. In addition, whereas both tBID and p53 induced release of cytochrome *c* from mitochondria, E4orf4 did not. The reasons for the discrepancies between these two studies could relate to differences between DNA transfection and infection with viral vectors, or to differences in cell density or culture conditions during the experiments. Nevertheless, the possibility exists that E4orf4 may be capable of inducing either caspase-independent or -dependent pathways. Thus, the uniqueness of the E4orf4 apoptosis pathway remains unresolved.

E4orf4 kills cancer cells selectively

Using transformed and primary normal rat cells, Shtrichman *et al.* (1999) suggested that E4orf4 may kill oncogenic but not normal rat cells. The latter were found to become more susceptible to killing by expression of certain activated oncogenes. Our group has examined over 40 human cancer cell lines representing most major classes of human tumors and found that E4orf4 induces massive cell death in all lines tested (Marcellus *et al.*, submitted). Interestingly, E4orf4 had little effect on about a dozen primary human cell types derived from various tissues. Cell death in these studies was measured by inhibition of cell growth, and thus E4orf4 killing was cancer cell-specific and not related to differences in proliferation. Studies were also conducted to determine the effect of E4orf4 on growth of human tumor xenografts in mice *in vivo*. E4orf4 was expressed in tumors by injection of a tetracycline-inducible E4orf4 adenovirus vector in mice given doxacyclin in drinking water. In the case of both C33A and H1299 human tumor cells, expression of E4orf4 caused a significant reduction in tumor size and progression. The effect was far more significant than that obtained using an adenovirus vector expressing p53, a leading candidate for human cancer gene therapy. Thus E4orf4 represents a major potential tool in cancer therapy, either individually or in combination with other genes or treatments.

Why does E4orf4 exhibit cancer cell specificity? The favored hypothesis is that expression of activated oncogenes or inactivated tumor suppressor genes in cancer cells supply essential signals that trigger cell death once the defective apoptotic signaling machinery in cancer cells has been reinstated by E4orf4. As such upstream signals are not present in normal cells, these cells are resistant to E4orf4 killing. It is also possible that all cancer cells contain higher levels of E4orf4 targets or that these targets or E4orf4 itself are more effectively modified post-translationally or localized in cancer cells. Further understanding of this specificity must await new insights into the mechanism of action of E4orf4.

Intracellular localization of E4orf4

E4orf4 is found in high quantities in cell nuclei following overexpression; however, significant amounts, especially at later times, are also present in the cytoplasm and associated with cytoplasmic components (MJ Miron, J Lavoie and PE Branton, unpublished). Although the localization of PP2A B subunits can vary during the cell cycle, the Bα subunit has been found in association with both microtubules and vimentin (Sontag *et al.*, 1995; Turowski *et al.*, 1999). B'(B56) family members have been found both in the cytoplasm and the nucleus (McCright *et al.*, 1996b). Preliminary studies using traditional immunofluorescence microscopy of cells expressing E4orf4 and various epitope-tagged B subunits indicated a high degree of apparent co-localization of E4orf4 with both

the Bα subunit and B′(B56) family members, especially in the cytoplasm (Shtrichman *et al.*, 2000). These results suggest that the primary targets for E4orf4-PP2A action may be cytoplasmic, although effects on nuclear targets cannot be ruled out. Further studies using E4orf4 proteins fused to specific targeting signals could be of value to better define the sites of action of E4orf4, and such studies are underway.

E4orf4, Src family kinases and extranuclear apoptosis

A recent study has demonstrated that E4orf4 can initiate caspase-independent extranuclear apoptosis through a dysregulation of Src family kinases (Lavoie *et al.*, 2000). Extranuclear apoptosis or anoikis generally occurs when cells lose integrin-mediated attachments to the extracellular matrix (ECM). These cell-ECM attachments promote the assembly of signaling molecules such as Src family kinases and focal adhesion kinase (FAK) to focal adhesion sites, where they then activate downstream survival pathways (Frisch and Ruoslahti, 1997; Giancotti, 1997). Extranuclear apoptosis occurs in three steps, the first being the release stage, which involves reorganization of focal adhesions and actin to give cells a rounder morphology, after which membrane blebbing and finally condensation into apoptotic bodies occurs (Mills *et al.*, 1999). While E4orf4 expressing cells are able to bind to ECM proteins, E4orf4 appears to interfere with downstream signals that promote cell spreading and survival. E4orf4 was found to associate and colocalize with Src family kinases and these complexes contain the PP2A catalytic subunit (Lavoie *et al.*, 2000). Whether the E4orf4-Src interaction is direct or mediated by other proteins remains to be determined. Src kinase activity appears to affect the distribution of E4orf4 in cells. About 30% of E4orf4 is normally associated with the cytoskeleton; however, in the presence of activated c-Src or v-Src this level increases 2–3-fold. The authors suggest that this massive translocation of E4orf4 may contribute to the triggering of cell death. Src activity also positively correlates with the amount of E4orf4-induced membrane blebbing and nuclear condensation. PP2, a chemical inhibitor specific to Src family kinases, significantly inhibited the number of apoptotic E4orf4-expressing cells. While E4orf4 does not affect the *in vitro* kinase activity of c-Src, it appears to modulate Src-dependent tyrosine phosphorylation of specific substrates with some having higher (FAK and paxillin) and others lower (cortactin) levels of tyrosine phosphorylation. Expression of E4orf4 also correlates with a dramatic relocalization of tyrosine phosphorylated proteins, actin fibers and cortactin (Lavoie *et al.*, 2000). The proposed model is that E4orf4 can interfere with Src/FAK signaling to promote the improper assembly of focal adhesions and ultimately the loss of survival signals. This effect would be accompanied by changes in the actin cytoskeleton, resulting in morphological and structural changes and membrane blebbing typical of apoptosis. It is still unclear how E4orf4 modulates Src kinase activity, becomes relocated in the presence of activated Src, or rearranges the actin cytoskeleton.

Mechanism of action of E4orf4

Interactions of E4orf4 with the Bα subunit (and/or possibly other members of the B55 family) are required for E4orf4-induced cell killing, and thus the major questions in determining the mechanism of action lie in defining the effects of E4orf4 on PP2A function and the targets of E4orf4-modified PP2A enzyme. Clearly E4orf4 can enhance the hypophosphorylation of several proteins, including c-Fos, E1A, E4F and SR factors, and alter c-Src-dependent phosphorylation patterns; however, it is neither clear if these proteins are direct targets for E4orf4-PP2A complexes, nor if these events are related to cell killing. Although binding low levels of PP2A activity by E4orf4 appears to be sufficient to kill some cells (Shtrichman *et al.*, 1999), our work with many cell types has clearly indicated that cell killing increases with increasing levels of E4orf4 expression (Marcellus *et al.*, submitted). These results suggest a stoichiometric relationship between E4orf4 and PP2A, possibly related to the ability of E4orf4 to locate appropriate PP2A holoenzymes, or a necessary shift in the global balance of normal and E4orf4-modified B55-PP2A. E4orf4 could alter the overall phosphatase activity of PP2A following binding to the Bα subunit, or more likely, modify the substrate specificity of such enzymes. Several mechanisms for reprofiling PP2A specificity could be possible, including: relocalization of PP2A by E4orf4 to preferred targets for cell death; modification of the Bα subunit to enhance activity against certain selective substrates; or binding of pro-apoptotic substrates to target them to PP2A. At present very little information exists to distinguish between these possibilities. In addition to B subunits, E4orf4 appears also to bind selective SR factors (Estmer-Nilsson *et al.*, 2001); however, several additional as yet unidentified proteins co-immunoprecipitate with E4orf4 (MJ Miron and PE Branton, unpublished). Identification of these species and further studies on the enzymatic activity of E4orf4-PP2A are required to provide new insights. In addition to these biochemical studies, downstream targets of E4orf4 may be elucidated through genetic approaches. In this regard, studies in yeast may be highly informative because of the availability of powerful genetic techniques in this organism.

E4orf4 in *Saccharomyces cerevisiae*

PP2A enzymes of *Saccharomyces cerevisiae* greatly resemble mammalian PP2A with respect to organization, substrate specificity, and sensitivity to inhibitors (Cohen *et al.*, 1989). A duplicated gene encodes two

forms of the catalytic C subunit, Pph21 and Pph22 (Sneddon *et al.*, 1990), that share 89% amino acid sequence identity. Both are at least 75% identical to the human C subunit peptide sequences (Costanzo *et al.*, 2000), but contain a unique acidic amino terminal region of unknown function (Ronne *et al.*, 1991). These subunits are dispensable for cell growth when deleted individually, but strains lacking both, as well as a related gene, *PPH3*, are non-viable (Ronne *et al.*, 1991). Temperature sensitive mutants have shown that the catalytic subunit is important for mitosis, cell wall integrity, and polarized growth (Evans and Stark, 1997). *TPD3* encodes the only A subunit, and like its mammalian counterpart it contains 15 non-identical repeats of 39 residues (van Zyl *et al.*, 1992), termed HEAT (huntingtin-elongation-Asubunit-TOR) motifs, that probably mediate protein-protein interactions (Groves *et al.*, 1999; Hemmings *et al.*, 1990). *tpd3* strains grow poorly and are both cold and heat sensitive, having morphological defects similar to *cdc55* cells at low temperatures and being defective for RNA polymerase III transcription at elevated temperatures (van Zyl *et al.*, 1992).

Only two B-type regulatory subunits exist in yeast and are encoded by *CDC55* and *RTS1*, representing the B and B′ families, respectively (Healy *et al.*, 1991; Shu *et al.*, 1997). Cdc55 is 47% identical and 61% similar to the human Bα subunit of PP2A (Costanzo *et al.*, 2000). *cdc55* deletion strains are cold sensitive and produce abnormally elongated cells due to defects in cytokinesis and/or septation, thus implicating PP2A in cell morphogenesis (Healy *et al.*, 1991). Cdc55 has also been shown to play a role in the cell cycle and appears to function at the spindle checkpoint as well as to be required for the proper regulation of Swe1 kinase (Wang and Burke, 1997; Yang *et al.*, 2000). Rts1 shares over 50% identity with mammalian B′ subunits (Costanzo *et al.*, 2000). *rts1* deletion strains are temperature sensitive for growth, with the majority of cells accumulating in G2/M with a single undivided nucleus (Shu *et al.*, 1997). Rts1 also regulates the global stress response in yeast (Evangelista *et al.*, 1996). Rts1 and Cdc55 are not functionally redundant PP2A subunits as Rts1 does not rescue cold sensitivity of *cdc55* cells, but actually enhances the morphological abnormalities of this mutant (Shu *et al.*, 1997).

S. cerevisiae provides a genetically tractable model system in which to study E4orf4 functions. E4orf4 expression was shown to promote irreversible growth arrest in yeast (Afifi *et al.*, 2001; Roopchand *et al.*, 2001), promoting an elongated morphology in cells arrested or delayed in the G2/M phase of the cell cycle, as demonstrated by FACS analysis and the presence of elevated Cdc28/Cdk1 kinase activity (Roopchand *et al.*, 2001; Kornitzer *et al.*, 2001). As with Bα in mammalian cells, E4orf4 interacts with the Cdc55 subunit and through this interaction recruits the entire active PP2A complex containing Pph21/22 and Tpd3 (Roopchand *et al.*, 2001). Evidence demonstrating the necessity of the B subunit for E4orf4 function in mammalian cells has been correlative thus far (Marcellus *et al.*, 2000; Shtrichman *et al.*, 1999). Using the yeast system a direct requirement for the B subunit has been clearly demonstrated, as whereas E4orf4 is fully toxic in an *rts1* deletion strain, the majority of E4orf4 toxicity is abolished in a *cdc55* deletion strain (Roopchand *et al.*, 2001; Kornitzer *et al.*, 2001); however, low levels of growth inhibition were still detected in the absence of Cdc55, suggesting that E4orf4 may have PP2A-independent functions (Roopchand *et al.*, 2001). In the absence of Cdc55, E4orf4 does not interact with the A and C subunits, nor does it associate with any detectable PP2A phosphatase activity, thus confirming the importance of Cdc55 for recruitment of PP2A and indicating that no interaction takes place with B′-like Rts1 subunits (Roopchand *et al.*, 2001). In wild-type yeast both class I (R81A/F84A) and class II (K88A) E4orf4 mutants induce a partial effect on cell growth similar to that of wild type E4orf4 in *cdc55* deletion strains, even though binding to Cdc55 was undetectable with the former and similar to wild type E4orf4 with the latter (Roopchand *et al.*, 2001). Taken together, these results again suggest that E4orf4 elicits Cdc55-independent effects. Further studies using yeast genetics should allow identification of E4orf4 downstream targets, and this information may shed light on the mechanism of induction of p53-independent killing of transformed cells.

Little is yet understood about the mechanism of E4orf4 toxicity in yeast, although one study may provide some insights. Induction of growth arrest in yeast was found to be associated with an accumulation of reactive oxygen species (Kornitzer *et al.*, 2001). Further, genetic studies using yeast mutants defective in mitosis have suggested an involvement of products required for mitosis, including Cdc28/Cdk1 and anaphase-promoting complex/cyclosome (APC/C). E4orf4 was found to inhibit APC/C activity, possibly through a direct interaction with APC/C, while enhancing that of Cdc28/Cdk1 (Kornitzer *et al.*, 2001). Although these observations need to be confirmed by other laboratories, they may represent a significant step in our understanding of E4orf4 action.

Future research and therapeutic potential of E4orf4

The discovery that B(B55) subunits of PP2A represent the primary targets in E4orf4-induced p53-independent apoptosis has provided a major insight into the mechanism of action of E4orf4. Nevertheless we understand little of the consequences of this interaction or whether or not the intracellular location of E4orf4-PP2A complexes is important. It will be important to determine what effect this interaction has on PP2A enzymatic activity, and to identify the specific targets involved in the onset of cell death. Cell death could be induced by increased or decreased PP2A-mediated dephosphorylation of relatively few substrates (perhaps those linked directly with cell survival or cell death pathways), or it may result from more global effects on a wide range of substrates in multiple pathways

causing an accumulated decline in cell viability. Death could also result indirectly as a consequence of changes in transcription, splicing, or translation induced by E4orf4. Thus analysis of changes in the patterns of gene and protein expression in response to E4orf4 using new technologies may be informative. It is also likely that genetic analyses to identify suppressors of E4orf4-induced killing using either mammalian or yeast systems will prove significant. Identification of additional E4orf4-binding proteins should also provide insights. All of these approaches are now underway in several laboratories.

Additional questions concern the identification of a possible second function of E4orf4 in cell killing, and whether E4orf4-induced cell death plays a role in the replication cycle of adenoviruses. As caspase activation and cytochrome *c* release are not stimulated by E4orf4 in at least some systems, E4orf4 should also provide insights into novel mechanisms of apoptosis. Further, the determination of the mechanism of the differential susceptibility of normal and cancer cells could provide insights for the development of new anti-cancer drugs.

E4orf4 has great potential in cancer therapy. A majority of human cancers contain mutations that inactivate p53, and many other cancers are probably defective in downstream components of the p53 pathway, including Arf. As many current therapies rely on p53-dependent cell killing, these treatments are only of limited value in these cases. E4orf4 kills a wide range of cancer cells independently of p53, and thus could be effective in all cancers, regardless of p53 status. Therefore the use of E4orf4 as a gene therapy agent or in the development of small molecule drugs that mimic E4orf4 function could yield highly effective and specific treatments for human cancer. We believe that many of these issues will be resolved in the near future.

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References

Afifi R, Sharf R, Shtrichman R and Kleinberger T. (2001). *J. Virol.*, **75**, 4444–4447.
Agostinis P, Derua R, Sarno S, Goris J and Merlevede W. (1992). *Eur. J. Biochem.*, **205**, 241–248.
Boivin D, Morrison MR, Marcellus RC, Querido E and Branton PE. (1999). *J. Virol.*, **73**, 1245–1253.
Bondesson M, Ohman K, Manervik M, Fan S and Akusjärvi G. (1996). *J. Virol.*, **70**, 3844–3851.
Bondesson M, Svensson C, Linder S and Akusjarvi G. (1992). *EMBO J.*, **11**, 3347–3354.
Bridge E, Medghalchi S, Ubol S, Leesong M and Ketner G. (1993). *Virology*, **193**, 794–801.
Calin GA, di Iasio MG, Caprini E, Vorechovsky I, Natali PG, Sozzi G, Croce CM, Barbanti-Brodano G, Russo G and Negrini M. (2000). *Oncogene*, **19**, 1191–1195.
Campbell KS, Auger KR, Hemmings BA, Roberts TM and Pallas DC. (1995). *J. Virol.*, **69**, 3721–3728.
Chiou SK and White E. (1997). *J. Virol.*, **71**, 3515–3525.
Cohen C, Schelling DL and Stark MJR. (1989). *FEBS Lett.*, **250**, 601–606.
Colella S, Ohgaki H, Ruediger R, Yang F, Nakamura M, Fujisawa H, Kleihues R and Walter G. (2001). *Int. J. Cancer*, **93**, 789–804.
Costanzo MC, Hogan JD, Cusick ME, Davis BP, Fancher AM, Hodges PE, Kondu P, Lengieza C, Lew-Smith JE, Lingner C, Roberg-Perez KJ, Tillberg M, Brooks JE and Garrels JI. (2000). *Nucleic Acids Res.*, **28**, 73–76.
Csortos C, Zolnierowicz S, Bako E, Durbin SD and DePaoli-Roach AA. (1996). *J. Biol. Chem.*, **271**, 2578–2588.
Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. (1997). *Cell*, **91**, 231–241.
de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ and Lowe SW. (1998). *Genes Dev.*, **12**, 2434–2442.
del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. (1997). *Science*, **278**, 687–689.
Engel DA, Hardy S and Shenk T. (1988). *Genes Dev.*, **2**, 1517–1528.
Estmer-Nilsson C, Petersen-Mahrt S, Durot C, Shtrichman R, Krainer AR, Kleinberger T and Akusjarvi G. (2001). *EMBO J.*, **20**, 864–871.
Evangelista CCJ, Rodriguez Torres AM, Limbach MP and Zitomer RS. (1996). *Genetics*, **142**, 1083–1093.
Evans DR and Hemmings BA. (2000). *Mol. Gen. Genetics*, **264**, 425–432.
Evans DR and Stark MJ. (1997). *Genetics*, **145**, 227–241.
Fernandes ER and Rooney RJ. (1997). *Mol. Cell. Biol.*, **17**, 1890–1903.
Ferrigno P, Langan TA and Cohen P. (1993). *Mol. Biol. Cell.*, **4**, 669–677.
Freyer GA, Katoh Y and Roberts RJ. (1984). *Nucleic Acids Res.*, **12**, 3503–3519.
Frisch SM and Ruoslahti E. (1997). *Curr. Opin. Cell. Biol.*, **9**, 701–706.
Frost JA, Alberts AS, Sontag E, Guan K, Mumby MC and Feramisco JR. (1994). *Mol. Cell. Biol.*, **14**, 6244–6252.
Giancotti FG. (1997). *Curr. Opin. Cell. Biol.*, **9**, 691–700.
Glenn GM and Eckhart W. (1995). *J. Virol.*, **69**, 3729–3736.
Gross A, McDonnell JM and Korsmeyer SJ. (1999). *Genes Dev.*, **13**, 1899–1911.
Groves MR, Hanlon N, Turowski P, Hemmings BA and Barford D. (1999). *Cell*, **96**, 99–110.
Halbert DN, Cutt JR and Shenk T. (1985). *J. Virol.*, **56**, 250–257.
Healy AM, Zolnierowicz S, Stapleton AE, Goebl M, DePaoli-Roach AA and Pringle JR. (1991). *Mol. Cell. Biol.*, **11**, 5767–5780.
Hemmings BA. (1997a). *Science*, **275**, 628–630.
Hemmings BA. (1997b). *Science*, **277**, 534.
Hemmings BA, Adams-Pearson C, Maurer F, Muller P, Goris J, Merlevede W, Hofsteenge J and Stone SR. (1990). *Biochemistry*, **29**, 3166–3173.
Hendrix P, Mayer-Jackel RE, Cron P, Goris J, Hofsteenge J, Merlevede W and Hemmings BA. (1993). *J. Biol. Chem.*, **268**, 15267–15276.

Herisse J, Rigolet M, de Dinechin SD and Galibert F. (1981). *Nucleic Acids Res.*, **9**, 4023–4042.
Janssens V and Goris J. (2001). *Biochem. J.*, **353**, 417–439.
Kanopka A, Muhlemann O and Akusjarvi G. (1996). *Nature*, **381**, 535–538.
Kanopka A, Mühlemann O, Petersen-Mahrt S, Estmer C, Ohrmalm C and Akusjärvi G. (1998). *Nature*, **393**, 185–187.
Kaplan JM, Armentano D, Scaria A, Woodworth LA, Pennington SE, Wadsworth SC, Smith AE and Gregory RJ. (1999). *J. Virol.*, **73**, 4489–4492.
Karin M and Hunter T. (1995). *Curr. Biol.*, **5**, 747–757.
Khew-Goodall Y, Mayer RE, Maurer F, Stone SR and Hemmings BA. (1991). *Biochemistry*, **30**, 89–97.
Kleinberger T. (2000). *Apoptosis*, **5**, 211–215.
Kleinberger T and Shenk T. (1993). *J. Virol.*, **67**, 7556–7560.
Korsmeyer SJ. (1999). *Cancer Res.*, **59**, 1693s–1700s.
Kremmer E, Ohst K, Kiefer J, Brewis N and Walter G. (1997). *Mol. Cell. Biol.*, **17**, 1692–1701.
Lavoie JN, Champagne C, Gingras MC and Robert A. (2000). *J. Cell. Biol.*, **150**, 1037–1056.
Lavoie JN, Nguyen M, Marcellus RC, Branton PE and Shore GC. (1998). *J. Cell. Biol.*, **140**, 637–645.
Livne A, Shtrichman R and Kleinberger T. (2001). *J. Virol.*, **75**, 789–798.
Munnervik M, Fan S, Ström AC, Helin K and Akusjärvi G. (1999). *Virology*, **256**, 313–321.
Marcellus RC, Chan H, Paquette D, Thirwell S, Boivin D and Branton PE. (2000). *J. Virol.*, **74**, 7869–7877.
Marcellus RC, Lavoie JN, Boivin D, Shore GC, Ketner G and Branton PE. (1998). *J. Virol.*, **72**, 7144–7153.
Marcellus RC, Teodoro JG, Wu T, Brough DE, Ketner G, Shore GC and Branton PE. (1996). *J. Virol.*, **70**, 6207–6215.
McCright B, Brothman AR and Virshup DM. (1996a). *Genomics*, **36**, 168–170.
McCright B, Rivers AM, Audlin S and Virshup DM. (1996b). *J. Biol. Chem.*, **271**, 22081–22089.
McCright B and Virshup DM. (1995). *J. Biol. Chem.*, **270**, 26123–26128.
Mills JC, Stone NL and Pittman RN. (1999). *J. Cell. Biol.*, **146**, 703–708.
Moreno CS, Park S, Nelson K, Ashby D, Hubalek F, Lane WS and Pallas DC. (2000). *J. Biol. Chem.*, **275**, 5257–5263.
Müller U, Kleinberger T and Shenk T. (1992). *J. Virol.*, **66**, 5867–5878.
Müller U, Roberts MP, Engel DA, Doerfler W and Shenk T. (1989). *Genes Dev.*, **3**, 1991–2002.
Nagata S. (1997). *Cell*, **88**, 355–365.
Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL and Roberts TM. (1990). *Cell*, **60**, 167–176.
Pallas DC, Weller W, Jaspers S, Miller TB, Lane WS and Roberts TM. (1992). *J. Virol.*, **66**, 886–893.
Querido E, Teodoro JG and Branton PE. (1997). *J. Virol.*, **71**, 3526–3533.
Ronne H, Carlberg M, Hu GZ and Nehlin JO. (1991). *Mol. Cell. Biol.*, **11**, 4876–4884.
Roopchand DE, Lee JM, Shahinian S, Paquette D, Bussey H and Branton PE. (2001). *Oncogene*, in press.
Roulston A, Marcellus RC and Branton PE. (1999). *Ann. Revs. Microbiol.*, **52**, 577–628.
Salvesen GS and Dixit VM. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 10964–10967.
Scheidtmann KH, Mumby MC, Rundell K and Walter G. (1991). *Mol. Cell. Biol.*, **11**, 1996–2003.
Shtrichman R and Kleinberger T. (1998). *J. Virol.*, **72**, 2975–2982.
Shtrichman R, Sharf R, Barr H, Dobner T and Kleinberger T. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 10080–10085.
Shtrichman R, Sharf R and Kleinberger T. (2000). *Oncogene*, **19**, 3757–3765.
Shu Y, Yang H, Hallberg E and Hallberg R. (1997). *Mol. Cell. Biol.*, **17**, 3242–3253.
Sneddon AA, Cohen PT and Stark MJ. (1990). *EMBO J.*, **9**, 4339–4346.
Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M and Mumby M. (1993). *Cell*, **75**, 887–897.
Sontag E, Nunbhakdi-Craig V, Bloom GS and Mumby MC. (1995). *J. Cell. Biol.*, **128**, 1131–1144.
Tanabe O, Nagase T, Murakami T, Nozaki H, Usui H, Nishito Y, Hayashi H, Kagamiyama H and Takeda M. (1996). *FEBS Lett.*, **379**, 107–111.
Tehrani MA, Mumby MC and Kamibayashi C. (1996). *J. Biol. Chem.*, **271**, 5164–5170.
Teodoro JG and Branton PE. (1997). *J. Virol.*, **71**, 1739–1746.
Teodoro JG, Shore GC and Branton PE. (1995). *Oncogene*, **11**, 467–474.
Turowski P, Myles T, Hemmings BA, Fernandez A and Lamb NJ. (1999). *Mol. Biol. Cell.*, **10**, 1997–2015.
van Zyl W, Huang W, Sneddon AA, Stark M, Camier S, Werner M, Marck C, Sentenac A and Broach JR. (1992). *Mol. Cell. Biol.*, **12**, 4946–4959.
Virtanen A, Gilardi P, Naslund A, LeMoullec JM, Pettersson U and Perricaudet M. (1984). *J. Virol.*, **51**, 822–831.
Voorhoeve PM, Hijmans EM and Bernards R. (1999). *Oncogene*, **18**, 515–524.
Walter G, Ruediger R, Slaughter C and Mumby M. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 2521–2525.
Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J and Evans GA. (1998). *Science*, **282**, 284–287.
Wang Y and Burke DJ. (1997). *Mol. Cell. Biol.*, **17**, 620–626.
Wei H, Ashby DG, Moreno CS, Ogris E, Yeong FM, Corbett AH and Pallas DC. (2001). *J. Biol. Chem.*, **276**, 1570–1577.
Whalen SG, Marcellus RC, Whalen A, Ahn NG, Ricciardi RP and Branton PE. (1997). *J. Virol.*, **71**, 3545–3553.
Wu J, Tolstykh T, Lee J, Boyd K, Stock JB and Broach JR. (2000). *EMBO J.*, **19**, 5672–5681.
Yamamoto K, Ichijo H and Korsmeyer SJ. (1999). *Mol. Cell. Biol.*, **19**, 8469–8478.
Yan Z, Fedorov SA, Mumby MC and Williams RS. (2000). *Mol. Cell. Biol.*, **20**, 1021–1029.
Yang H, Jiang W, Gentry M and Hallberg RL. (2000). *Mol. Cell. Biol.*, **20**, 8143–8156.
Yang SI, Lickteig RL, Estes R, Rundell K, Walter G and Mumby MC. (1991). *Mol. Cell. Biol.*, **11**, 1988–1995.
Zhu H, Fisk HA, Yaffe MP, Mahajan N, Herman B and Reed JC. (1996). *Mol. Cell. Biol.*, **16**, 6494–6508.
Zhao Y, Boguslawski G, Zitomer RS and DePaoli-Roach AA. (1997). *J. Biol. Chem.*, **272**, 8256–8262.
Zolnierowicz S, Csortos C, Bondor J, Verin A, Mumby MC and DePaoli-Roach AA. (1994). *Biochemistry*, **33**, 11858–11867.
Zolnierowicz S, V.H.C., Andjelkovic N, Cron P, Stevens I, Merlevede W, Goris J and Hemmings BA. (1996). *Biochem. J.*, **317**, 187–194.

Exhibit C

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Conditionally Replicating Adenoviruses Kill Tumor Cells via a Basic Apoptotic Machinery-Independent Mechanism That Resembles Necrosis-Like Programmed Cell Death

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Conditionally replicating adenoviruses (CRAds) represent a promising class of novel anticancer agents that are used for virotherapy. The E1AΔ24 mutation-based viruses, Ad5-Δ24 [CRAd(E3−); E3 region deleted] and infectivity-enhanced Ad5-Δ24RGD [CRAd(E3+)] have been shown to potently eradicate tumor cells. The presence of the E3 region in the latter virus is known to improve cell killing that can be attributed to the presence of the oncolysis-enhancing Ad death protein. The more precise mechanism by which CRAds kill tumor cells is unclear, and the role of the host cell apoptotic machinery in this process has been addressed only in a limited way. Here, we examine the role of several major apoptotic pathways in the CRAd-induced killing of non-small-cell lung cancer H460 cells. As expected, CRAd(E3+) was more potent than CRAd(E3−). No evidence for the involvement of the p53-Bax apoptotic pathway was found. Western blot analyses demonstrated strong suppression of p53 expression and unchanged Bax levels during viral replication, and stable overexpression of human papillomavirus type 16-E6 in H460 cells did not affect killing by both CRAds. CRAd activity was also not hampered by stable overexpression of anti-apoptotic Bcl2 or BclXL, and endogenous Bcl2/BclXL protein levels remained constant during the oncolytic cycle. Some evidence for caspase processing was obtained at late time points after infection; however, the inhibition of caspases by the X-linked inhibitor of apoptosis protein overexpression or cotreatment with zVAD-fmk did not inhibit CRAd-dependent cell death. Analyses of several apoptotic features revealed no evidence for nuclear fragmentation or DNA laddering, although phosphatidylserine externalization was detected. We conclude that despite the known apoptosis-modulating abilities of individual Ad proteins, Ad5-Δ24-based CRAds trigger necrosis-like cell death. In addition, we propose that deregulated apoptosis in cancer cells, a possible drug resistance mechanism, provides no barrier for CRAd efficacy.

Conditionally replicating adenoviruses (CRAds) represent a promising class of biologic agents designed to selectively replicate in and lyse cancer cells, also known as virotherapy (for reviews, see references 2, 23, and 37). Tumor specificity of CRAds has been achieved by modifying a viral gene(s) important for efficient viral replication in normal cells but not in tumor cells that possess complementing genetic defects.

Two types of CRAds can be distinguished, in which either the adenoviral genome is modified by a specific mutation or deletion or tumor-specific promoters are inserted to drive the expression of essential viral genes. For example, Ad5-Δ24 contains a partial deletion in the CR2 domain of the pRb-binding protein E1A (dl922-947) that is complemented in pRb-deficient tumor cells (13). An integrin-directed infectivity-enhanced variant, CRAdΔ24RGD, which is currently being evaluated in clinical trials, demonstrated effective killing of different cancer cells in vitro and in vivo (3, 29, 46, 47).

In animal studies and in the clinic, CRAds appear to be safe and well tolerated; however, their antitumor activity as a single agent is modest, and improvement of the therapy is required (14, 24, 40). Strategies currently explored to enhance the efficacy of these agents include the combined use of CRAds with conventional therapies and the generation of armed CRAds containing cytotoxic payloads. In this respect, the possibility that interactions between the viral genome and host cell factors will determine adenoviral oncolytic efficacy has been left relatively unexplored (28) for wild-type Ad (wtAd) and has not been addressed for CRAdΔ24 variants. Ads normally infect cells of the respiratory tract; however, they are redirected in virotherapy to infect and kill tumor cells. Tumor cells are likely to possess intrinsic genetic differences compared to normal host cells that may also exist among different tumor types, which could either facilitate or hamper CRAd replication and cell killing. Such mechanisms may provide an explanation for observed discrepancies in CRAd efficacy in addition to the known distinct infection efficiencies.

Apoptosis is a specific form of programmed cell death (PCD) that is characterized by several morphological changes that are most prominently visible in the nucleus, including chromosome condensation and nuclear shrinkage and fragmentation. The caspases, members of the family of aspartic acid-specific cysteine-proteases, are the executioners of apoptosis and essential for the disassembly of the cell (for reviews, see references 9, 20, 45, and 48). Two main apoptotic pathways have been identified that induce caspase-dependent apoptosis upon exposure to various stimuli. The intrinsic pathway can be triggered by cytotoxic agents, for example, and involves the destabilization of mitochondria leading to activation of caspase-9. The extrinsic pathway is initiated via death receptors, such as Fas/CD95, that activate caspase-8. Both caspase-8 and

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caspase-9 can activate the downstream or executor caspase-3, -6, and -7. The activity of caspases can be regulated by members of the inhibitor of apoptosis (IAP) family, such as X-linked IAP (XIAP) (54). The Bcl-2 family of proteins comprise antiapoptotic and proapoptotic members that are key regulators of the mitochondrial pathway (1, 27, 41). Proapoptotic members, including Bax, can induce mitochondrial instability leading to the release of cofactors that allow the activation of caspase-9 assembled in the apoptosome. The activation of the basic apoptotic machinery as briefly outlined above is considered to largely determine the success of cancer therapies, and obstruction of this apoptotic machinery in cancer cells may cause resistance to treatment.

Ads have a rather complex infrastructure in which the viral genes produce numerous proteins that prevent the death of infected cells early after infection and other proteins that favor cell death at later stages (6, 28, 33, 38, 42, 52). Several viral encoded proapoptotic and antiapoptotic proteins are known to maintain temporal control of the Ad on the host cell.

Most notably, E1B-19KD (an Ad Bcl2 homologue), E1B-55KD in complex with E4orf6, and the E3-10.4KD and E3-14.5KD proteins, which are encoded by early transcribed genes, act as antiapoptotic proteins and are responsible for the prevention of premature oncolysis or cause immune suppression, while E1A-12S, E1A-13S, E4orf4, and E3-11.6KD (Ad death protein [ADP]) act as proapoptotic proteins when tested individually. In addition, the mechanism responsible for the oncolysis-enhancing effect of ADP is largely unknown (32, 50). Thus, during Ad infection, the viral proteins act in concert with several host cell-encoded key apoptotic regulators in a sort of cell death-balancing act, leading to the timed delay of cell death and allowing the efficient replication and generation of viral offspring.

Despite the known apoptosis-regulatory function of individual Ad genes, it is currently unknown whether the disruption of cancer cells at the last stage of CRAd infection, named oncolysis, employs the basic apoptotic machinery of the host cell. To explore this, we set out to investigate the role of the basic apoptotic machinery during the CRAdΔ24-induced killing of non-small-cell lung cancer (NSCLC) H460 cells. The involvement of several main apoptotic pathways was studied, including p53, Bcl2, and caspase-mediated cell death, and the morphological and biochemical features associated with CRAdΔ24-induced cell death were examined. Interestingly, CRAd-induced cell death can be classified as a necrosis-like PCD that bypasses the apoptotic machinery of the host cell irrespective of the presence or absence of the E3 region (ADP), suggesting that deregulated apoptotic pathways in cancer are unlikely to have a negative impact on CRAd-induced cell killing.

MATERIALS AND METHODS

Cell culture and treatment. The human NSCLC NCI H460 parental cell line ($p53^+$ Rb wt^+ $p16INK4^-$) (22); the previously generated derivatives H460Bcl2, H460BclXL, and H460XIAP; these derivatives' empty vector controls H460\PEFPGK3 and H460\pcDNA3 (11); and H460–human papillomavirus type 16 (HPV16)-E6 (designated H460HPV16-E6) (53) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Breda, The Netherlands), 50 IU of penicillin/ml, 50 μg of streptomycin/ml, and 1 μg of puromycin/ml or 200 μg of Geneticin/ml, depending on the cell line, and grown at 37°C in a humidified air with 5% CO_2. The cells were tested routinely for the absence of mycoplasma before use, and overexpression of Bcl2, BclXL, and XIAP proteins was confirmed by immunohistochemistry prior to use in the experiments. For apoptosis activation, cells were treated with 25 ng of anti-Fas activating antibody (clone CH11; Upstate Biotechnology, Inc., Lake Placid, N.Y.)/ml together with 0.5 μg of cycloheximide (CHX; Sigma, St. Louis, Mo.)/ml for 16 h or with 14 μM cisplatin (CDDP; Bristol-Myers Squibb, Woerden, The Netherlands) for 3 days. Caspase activation was inhibited by exposure of cells to the pan-caspase inhibitor zVAD-fmk at 100 μM (Enzyme Systems Products, Livermore, Calif.).

CRAd infection and viability measurement. AdΔ24 [CRAd(E3−)] and AdΔ24RGD [CRAd(E3+)] harbor a 24-bp deletion in the pRb-binding CR2 domain in the E1A region; the latter contains the E3 region (E3+) and harbors an RGD motif cloned in the fiber knob that enhances infectivity in a wide range of cancer cells (47). Cells cultured to near-confluence in 96-well plates were incubated with different multiplicities of infection (MOIs) (0.001 to 100 PFU/cell) of CRAd(E3−) or CRAd(E3+) in growth medium (50 μl/well) at 37°C. Two hours postinfection, another volume of virus-free medium was added. Cell viability was measured within 7 days postinfection with WST-1 reagent (Roche Diagnostics, Mannheim, Germany). Briefly, the culture medium was removed and replaced by 100 μl of 10% WST-1 in culture medium.

Depending on cell type and density, the formation of the formazan dye was allowed to proceed for 30 to 60 min at 37°C, and the A_{450} was measured with a model 550 microplate reader (Bio-Rad Laboratories, Hercules, Calif.). The percentage of growth (WST-1 conversion) of treated cells was expressed as a percentage of the conversion by uninfected control cells after subtraction of background values of WST-1 incubated in the absence of cells. Alternatively, the oncolytic potential of the CRAds in relation to cell viability was determined by crystal violet staining. Cells were washed with phosphate-buffered saline (PBS) (10.9 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 8.2 g of NaCl/liter), fixed for 10 min at room temperature (RT) in 4% (vol/vol) formaldehyde in PBS, and stained using 10 g of crystal violet dye/liter in 70% (vol/vol) ethanol for 20 min at RT. After being washed several times with water, the culture plates were air dried prior to imaging.

Western blotting. Cells were lysed at 24, 48m and 72 h postinfection as indicated in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% sodium dodecyl sulfate, 0.5% sodium deoxycholate [Fluka Biochemika, Buchs, Switzerland], 1% Igepal [NP-40; Sigma], and 1 mM PEFA block protease inhibitors mixture [Roche Diagnostics]) on ice and stored at −80°C for further use. After determining sample protein concentrations by the bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Rockfort, Ill.), 20 μg of each sample was separated on a sodium dodecyl sulfate–12.5%polyacrylamide gel electrophoresis gel. Thereafter, the proteins were blotted on a polyvinylidene difluoride membrane (Bio-Rad). The membrane was rinsed in blocking solution containing 5% nonfat milk in TBST (0.1% Tween 20, 150 mM NaCl, and 10 mM Tris-HCl [pH 8]). The following first antibodies, dissolved in 5% bovine serum albumin–TBST, were used: anti-human p53 monoclonal antibody (MAb) (dilution 1:1,000; DAKO, Glostrup, Denmark), anti-Bax MAb (dilution 1:250; BD Transduction Laboratories, Sparks, Md.), rabbit polyclonal anti-BclXL (dilution 1:250; DAKO Diagnostics, Mississauga, Ontario, Canada), anti-Bcl2 MAb (dilution 1:2,000; DAKO), rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP) (dilution 1:2000; Roche, Almere, The Netherlands), anti-caspase-8 MAb (dilution 1:2,000; Immunotech, Prague, Czech Republic), rabbit anti-caspase-9 (dilution 1:2,000; PharMingen, San Diego, Calif.), anti-caspase-3 MAb (dilution 1:1,000; BD Transduction Laboratories), and anti-β-actin MAb (dilution 1:7,500; Sigma).

After incubation for 1 to 2 h with the primary antibody and washing in TBST, the blots were incubated with peroxidase-conjugated goat anti-rabbit (dilution 1:5,000) or rabbit anti-mouse (dilution 1:2,000) IgG secondary antibody (DAKO). For chemoluminescence detection, blots were immersed in enhanced chemiluminescence solution and exposed to hyperfilm (Amersham Pharmacia UK, Ltd., Buckinghamshire, United Kingdom).

Apoptosis detection assays. For DNA fragmentation analysis, 5×10^5 cells were harvested 72 h postinfection, and DNA was extracted as described previously (12). In brief, cells were pelleted and lysed with 30 μl of lysis buffer containing 20 mM EDTA, 100 mM Tris (pH 8.0), 0.8% sodium lauryl sarcosinate, and 5 mg of proteinase K/ml. After 2 h of incubation at 50°C, 0.2 mg of RNase A/ml was added, and the lysate was incubated for another 30 min at 37°C, prior to electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.2 μg/ml). Nuclear morphology was examined in treated or untreated cells cultured on coverslips that were washed once with PBS before incubation in the fixative (3.7% formaldehyde in PBS) for 30 min at RT, stained with Hoechst 33342 (Sigma) for 1 h at RT, and mounted with Vectashield (Vector Laboratories Inc., Burlingame, Calif.). Stained nuclei were visualized at a 40× magnification under UV light. Phosphatidylserine (PS) externalization was determined after exposure to the phospholipid-binding protein Annexin V, according to the manufacturer's protocol (Roche). In brief, cells cultured on coverslips were incubated with fluorescein-labeled Annexin V and propidium iodide (PI)-containing buffer (Roche) for 15 min at RT. Annexin V and PI staining were visualized at a

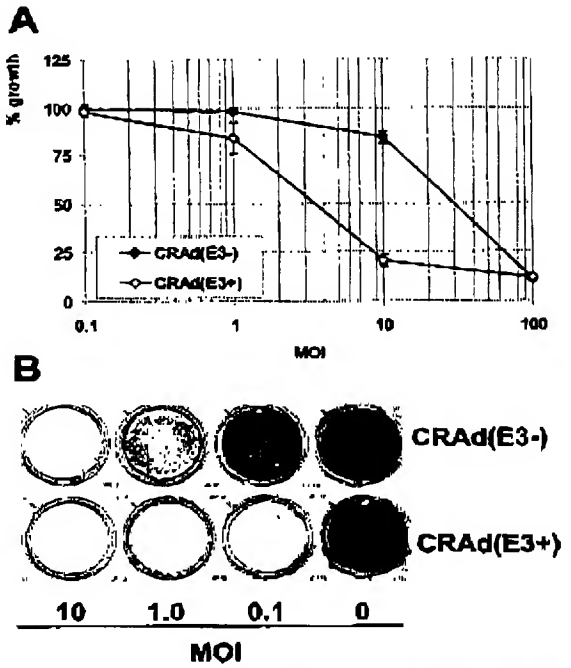

FIG. 1. Infectivity-enhanced AdΔ24RGD[CRAd(E3+)] is more potent in killing H460 cells than AdΔ24[CRAD(E3−)]. The viability of H460 cells was determined by the WST-1 assay, with three experiments. Data show means ± standard deviation (A) and crystal violet staining (B) at 5 and 11 days postinfection, at different MOIs.

63× magnification under blue and green light, respectively, with an inverted IMIRB/E fluorescence microscope (Leica, Heidelberg, Germany) using Leica O500MC Quantimet software, version 1.01 (Leica Cambridge, Ltd., Cambridge, United Kingdom).

RESULTS

CRAd-induced killing of H460 cells. To determine the mode of cell death induced by CRAds, we used two variants: E3 region-deleted virus, CRAd(E3−), and CRAd(E3+), containing the E3 region and possessing integrin-mediated enhanced infectivity (29). In this study, we compared the mechanism of cell killing by these two CRAds, assuming that RGD targeting only leads to enhanced infection efficiency without affecting the underlying mechanism of cell killing. For a model system, we employed the NSCLC H460 cell line. First, we assessed the oncolytic effect of the CRAds in H460 cells by determining cell viability by WST-1 (Fig. 1A) and crystal violet staining assays (Fig. 1B) at 5 and 11 days, respectively, after infection with different MOIs. As expected, both viruses showed MOI-dependent toxicity with CRAd(E3+) that was more potent than with CRAd(E3−) in killing H460 cells; 50% inhibitory concentration values were 3.5 and 30 PFU/cell, respectively.

p53-Independent cell killing by CRAds in H460 cells. To investigate the role of p53 in mediating cell death triggered by CRAds in H460 cells, the expression of p53 was examined by Western blotting in extracts derived from CRAd-infected cells that were harvested at different time points after infection (Fig. 2A). The levels of p53 progressively decreased within 2 days after CRAd(E3−) infection, which can be attributed to E1B-55KD-mediated degradation (18, 36). The presence of the E3 region accelerated the reduction in p53 levels that may also be caused by accelerated internalization via the integrin-targeting motif RGD. The expression of the p53-inducible proapoptotic Bcl2 family member Bax was also studied, revealing a time-dependent decrease after CRAd(E3−) infection. The presence of the E3 region augmented CRAd-induced Bax reduction at 24 h postinfection, and levels remained low at later time points. As a control, H460 cells were treated with CDDP, which resulted in the induction of Bax and a less appreciable accumulation of p53 (Fig. 2A), which is in line with the expected activation of a p53-dependent route to apoptotic cell death. Activation of a p53-independent death receptor pathway with an agonistic Ab directed against the Fas receptor caused a decrease in the levels of p53 and Bax.

To further assess the role of p53 in CRAd-induced cell death, H460 cells stably transfected with HPV16-E6, which possess reduced levels of p53 caused by HPV16-E6-mediated degradation of p53 (Fig. 2B), were infected with different MOIs of CRAd(E3+). Cell viability was measured at 7 days postinfection and was compared to CRAd toxicity in empty vector-transfected p53-expressing control cells (H460-neo) (Fig. 2C). The E6-dependent inhibition of p53 did not influence the oncolytic effect of this CRAd. Together, these data indicate that the cell killing effect of both tested CRAds is not dependent on the activation of p53-dependent apoptosis.

Cell death induced by CRAds is triggered independent of caspases and Bcl2. Next, the contribution of the basic apoptotic machinery to CRAd-induced cell death was evaluated more directly. H460 cells were infected with the two CRAds at an MOI of 25, and the processing of procaspases-9, -8, and -3 and the caspase substrate PARP was determined by Western blotting at various days postinfection. Figure 3A shows that both CRAds produced some PARP cleavage at 24 and 48 h after infection, followed by a decrease in both unprocessed PARP (116 kDa) and cleaved PARP (89 kDa) at 3 days postinfection. The activation of caspases was studied by monitoring the decrease in the band representing the procaspase form that is indicative for caspase activation. Apart from a small decrease in the procaspase bands at 3 days postinfection, there is no clear indication of caspase processing in contrast to the strong activation observed during Fas-induced apoptosis (Fig. 3A) that was used as a positive control for death receptor and caspase-dependent apoptosis. Subsequently, the involvement of the anti-apoptotic Bcl2 family members Bcl2 and BclXL, key regulators of the mitochondrial apoptotic pathway, was studied during CRAd-induced cell death. In Western blotting experiments, the expression levels of Bcl2 and BclXL did not decrease (Fig. 3B); together with the above-demonstrated lack of increase in Bax levels (Fig. 1A), these findings are indicative for the lack of participation of the mitochondrial pathway in mediating cell killing by these CRAds.

To further test the role of caspases and Bcl2 in CRAd-triggered cell death, we used a panel of H460-derived stable transfectants overexpressing Bcl2, BclXL, and XIAP, the latter an inhibitor of caspase-9- and caspase-3-dependent apoptosis (54). These cell lines have been generated previously and shown to be potent in inhibiting mitochondria- and caspase-dependent apoptosis in H460 cells (11). The stable transfec-

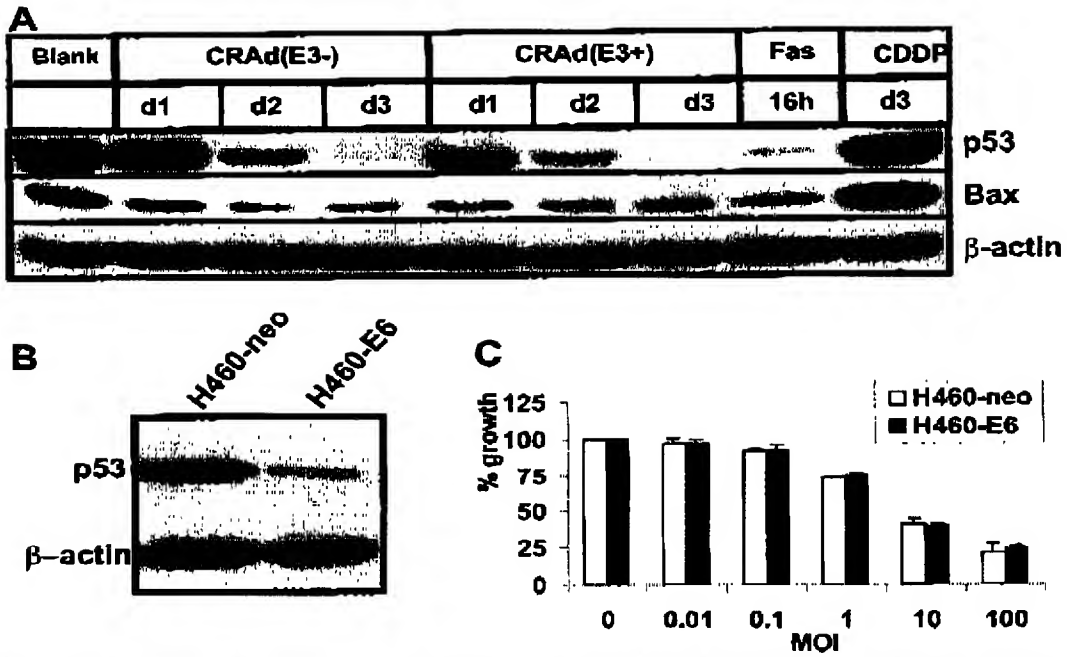

FIG. 2. The p53-Bax apoptotic pathway is not involved in CRAd(E3+)- or CRAd(E3−)-induced cell killing of H460 cells. Western blot analyses of p53 and Bax expression in H460 cells infected with CRAd(E3+) or CRAd(E3−) at an MOI of 25 at different time points after infection are shown. As a control, H460 cells were treated with CDDP or with FasL+CHX (Fas). β-Actin serves as a control for loading (A). The reduced expression level of p53 in stable HPV16-E6-overexpressing H460 cells (H460-E6) was confirmed (B). CRAd-induced cell killing at different MOIs was examined in H460-E6 cells and in empty vector-transfected control H460 cells (H460-neo) at 7 days after infection by WST-1 assays. The results obtained for CRAd(E3+) are shown and were similar to those for CRAd(E3−) (C). Values are means ± standard deviation of three experiments.

tants were tested for their sensitivity to the oncolytic effect of CRAd(E3+) when compared to parental H460 cells and vector-transfected control H460 cells (Fig. 3C, H460\PEFPGK3 and H460\pcDNA3). No protection was observed by the overexpression of Bcl2, BcXl, or XIAP, and similar results were found for CRAd(E3−) (data not shown).

As a final test to examine the possible involvement of caspases in CRAd-induced cell death, H460 cells were cotreated with the broad caspase inhibitor zVAD-fmk at a concentration of 100 μM, which completely protected against the apoptotic effect of FasL plus CHX (FasL+CHX) (Fig. 3D). In line with the above, CRAd activity was not suppressed in a detectable way by zVAD-fmk administration, further confirming that caspases are not involved in the execution of CRAd-induced cell death.

Analyses of biochemical and morphological features of apoptosis in H460 cells undergoing CRAd-induced cell death. Finally, CRAd-dependent changes in several parameters of apoptosis were examined. Nuclear morphology was studied by staining H460 cells with Hoechst 33342 at 3 days after infection with both CRAds at an MOI of 25 in the presence or absence of 100 μM zVAD-fmk to assess caspase-dependent phenomena (Fig. 4A). Regardless of the presence of the E3 region, both CRAds seemed to cause some swelling of the nuclei, and regions with more intense staining (i.e., a speckled appearance) were observed that were not affected by zVAD-fmk treatment. In contrast, FasL+CHX exposure clearly induced chromosome condensation, nuclear shrinkage, and/or fragmentation that could be reverted to normal by cotreatment with 100 μM zVAD-fmk, indicative of caspase-dependent apoptosis.

DNA fragmentation assays were also performed at 3 days after infection with both CRAds. Contrary to the pronounced DNA fragmentation found in FasL+CHX-positive control cells, no DNA fragmentation was observed in CRAd(E3+)- or CRAd(E3−)-infected cells (Fig. 4B). Caspase inhibition by 100 μM zVAD-fmk completely protected against FasL+CHX-induced DNA laddering.

The last marker of apoptosis that we examined was the loss of phospholipid symmetry and externalization of PS residues in cells with an intact membrane that is indicative for apoptosis (10, 21) and which was determined by AnnexinV staining within 2 days after CRAd infection. The cells were costained with PI in order to discriminate apoptotic cells (Annexin V positive and PI negative) from necrotic ones (Annexin V and PI positive due to membrane permeabilization). The results obtained were similar for both CRAds and are only shown for CRAd(E3+), which induced a somewhat more rapid PS externalization than CRAd(E3−), in line with its faster rate of oncolysis. Untreated cells remained negative for Annexin V staining for the duration of the experiment (Fig. 4C). The majority of CRAd-infected cells showed Annexin V-positive membrane staining in the absence of PI staining. In a small

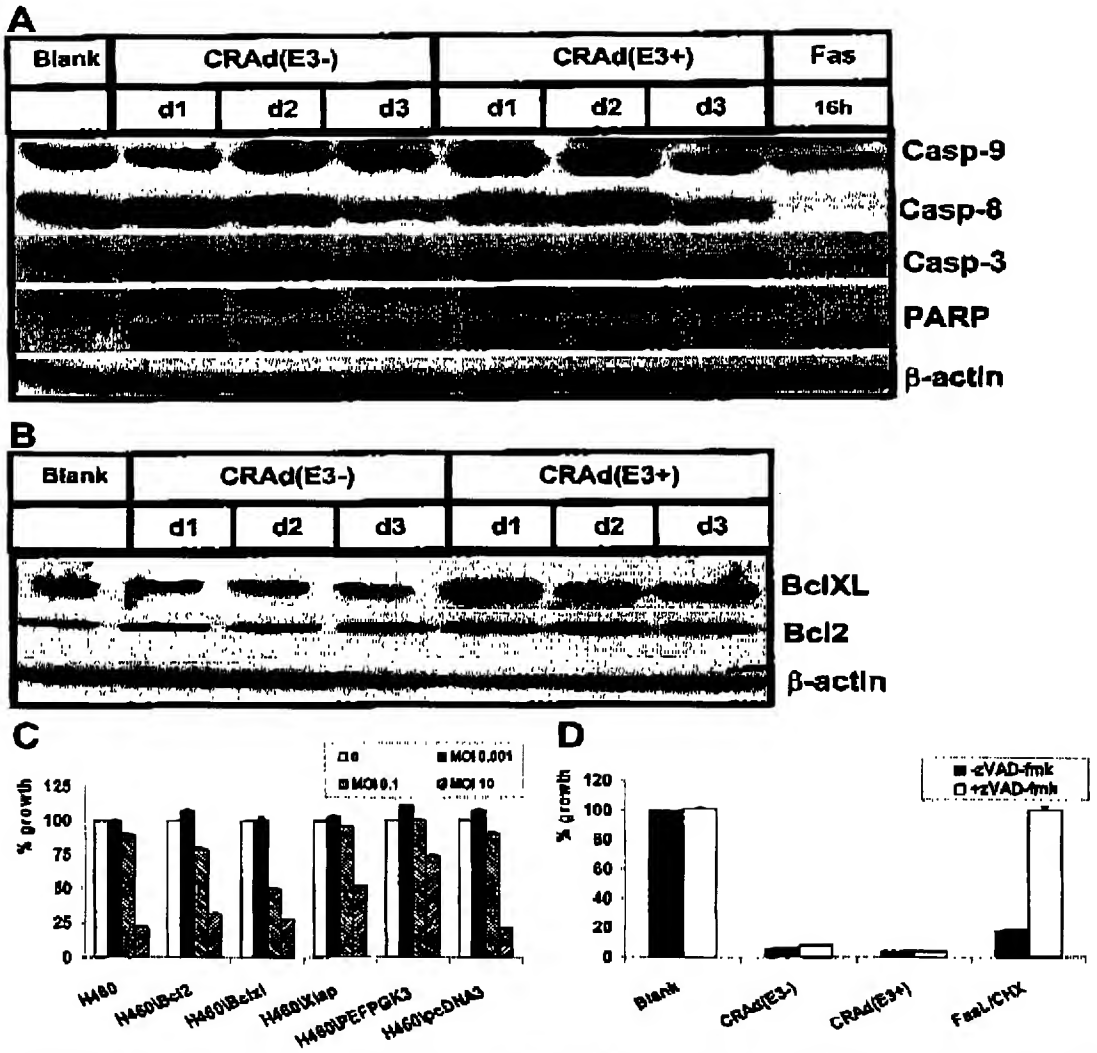

FIG. 3. CRAd(E3±)-induced cell death is not mediated by caspases and is not dependent on the activation of the mitochondrial apoptotic pathway regulated by Bcl2. H460 cells were infected at an MOI of 25, and cell extracts were made at different times postinfection and subjected to Western blotting. As a control, H460 cells were treated with agonistic Fas Abs in combination with CHX (Fas). (A) The expression levels of unprocessed procaspase-9, -8, and -3 were assessed together with the cleavage of caspase substrate PARP. (B) Bcl2 and BclXL expression was also determined. (C) CRAd(E3+)-induced cell killing in H460-derived stable transfected cell lines overexpressing antiapoptotic Bcl2 and BclXL or the caspase-inhibitor XIAP when compared to the empty vector controls H460\PEFPGK3 and H460\pcDNA3 was studied. Different MOIs were used, and WST-1 activity was measured at 4 days postinfection. Similar results were obtained for CRAD(E3−). (D) Cotreatment with the broad caspase inhibitor zVAD-fmk failed to protect H460 cells from CRAd(E3±)-induced cell death, whereas it was effective in protecting against apoptosis induced by FasL+CHX cells that served as a positive control. Values are means ± standard deviation of three experiments.

portion of CRAd-infected cells, PS externalization was accompanied by PI staining; this may represent a minority of cells that became leaky during virus propagation. Coexposure with zVAD-fmk did not alter the Annexin V staining pattern in the infected cells. FasL+CHX-treated cells displayed Annexin V-positive and PI-negative cells, and PS exposure was completely prevented by zVAD-fmk (Fig. 4C). This indicates that CRAds trigger PS externalization via a caspase-independent mechanism, in contrast to FasL+CHX treatment, which induces caspase-dependent PS exposure.

DISCUSSION

The eradication of cancer cells by gene therapy approaches that are based on the molecular characteristics of cancer cells provides a promising new therapeutic platform. In this respect, CRAds represent rationally designed agents for the selective

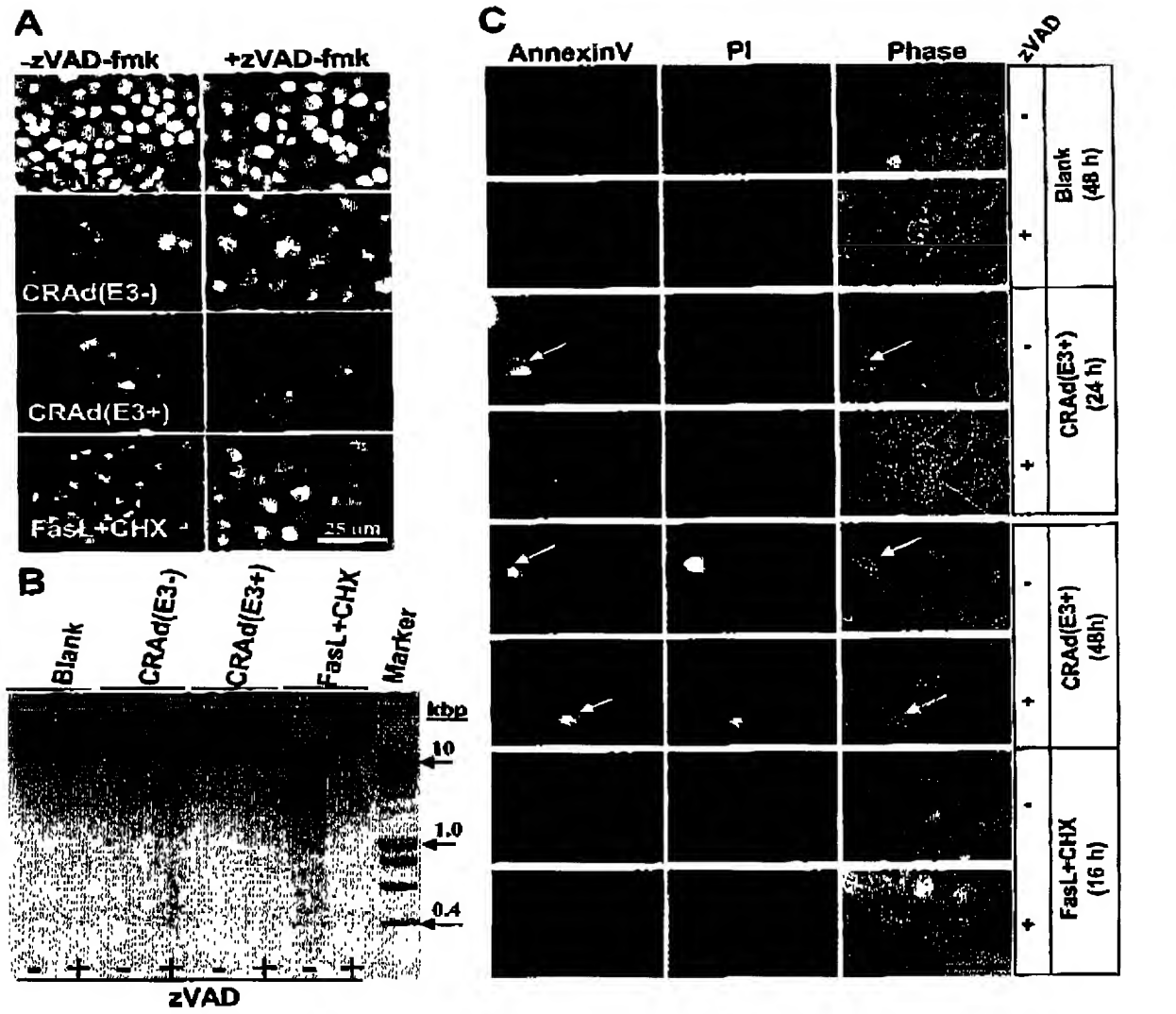

FIG. 4. Lack of apoptotic features in H460 cells undergoing cell death triggered by CRAd(E3+) and CRAd(E3−). (A) H460 cells were infected with both CRAds at an MOI of 25 alone or in combination with 100 μM zVAD-fmk for 2 days. As a control, cells were exposed for 16 h to FasL+CHX with or without zVAD-fmk. Nuclei were stained with Hoechst 33342 to visualize chromatin condensation, nuclear shrinkage, or fragmentation that are markers for apoptosis by immunofluorescence microscopy. CRAd-infected cells did not display nuclear apoptotic features, in contrast to Fas+CHX-exposed cells that show condensed and fragmented nuclei reversible by zVAD-fmk. (B) DNA fragmentation was also analyzed, revealing no apoptotic DNA laddering in CRAd-infected cells, whereas FasL+CHX treatment results in clear DNA smearing. (C) Annexin V staining caused by PS externalization was examined 1 and 2 days postinfection and in FasL+CHX-treated H460 cells with and without zVAD-fmk. Cells were cotreated with PI to allow the distinction between Annexin V staining in the presence or absence of an intact membrane detected by immunofluorescence microscopy; the phase-contrast image is also shown (Phase). A minor portion of CRAd-infected cells were PI positive (indicated by arrows). Annexin V staining is caspase independent in CRAd-infected cells and caspase dependent when induced by FasL+CHX. Results are shown for CRAd(E3+)-infected cells; similar findings were obtained for CRAd(E3−)-infected cells.

killing of cancer cells, while leaving normal cells intact. Despite exciting laboratory results with CRAds, several practical obstacles need to be overcome before virotherapy can fulfill its goals in the clinic.

In this study, we focused on the possibility that the host cell apoptotic machinery may either facilitate or suppress CRAd-induced cell killing in cancer cells, as this may cause resistance to CRAds and lead to intratumor variation in CRAd efficacy. In various cellular and biochemical assays, we showed that CRAd(E3−) and CRAd(E3+) kill H460 NSCLC cells independent from the basic apoptotic machinery. At late time points after infection, some evidence for procaspase processing

TABLE 1. Summary of apoptotic features found in H460 cells infected with AdΔ24-based CRAds and mode of cell death

Cellular and molecular changes	Cell death				Induced cell death	
	Apoptosis	Apoptosis-like PCD	Necrosis-like PCD	Necrosis	CRAds-induced oncolysis	FasL+CHX
Chromatin condensation	+	+	−	−	+[a]	+
DNA laddering	+	−/+	−	−	−	+
Cytoplasmic shrinkage	+	−/+	−/+	−	−	+
PS exposure	+	+	+	−	+	+
Caspase activation	+	−/+	−/+	−/+	−	+

[a] Chromatin condensation occurs but is distinct from "apoptotic condensation" (see text).

was found (Fig. 3); however, the pan-caspase-inhibitor zVAD-fmk and overexpressed XIAP did not influence CRAd-mediated H460 cell killing, indicating that the observed caspase cleavage is a cophenomenon and not instrumental for CRAd toxicity. In line with this, the small proportion of cleaved PARP may reflect to some extent the apoptosis-regulatory activity of the adenoviral proteins in preventing the cell from dying early before completion of the reproductive viral cycle. Rather than inducing PARP cleavage, PARP expression fades away at 3 days postinfection, which is suggestive of caspase-independent cell death. The lack of effect of caspase inhibition by zVAD-fmk on cell death triggered by these CRAds was also observed in NSCLC A549 and SW1574 cells (unpublished data).

The apparent lack of involvement of the basic apoptotic machinery in mediating CRAd-induced cell death was confirmed in H460 cells overexpressing Bcl2 or BclXL that failed to protect against the oncolytic effect of CRAds. In this context, the presence or absence of the E3 region did not affect the mode of cell death, in agreement with the observed nonapoptotic cell death induced by ADP (6, 50).

Previously, it has been speculated that ADP that is expressed in large amounts at late stages after infection facilitates oncolyses in a stoichiometric manner by targeting the nuclear membrane where it either binds and/or deactivates antiapoptotic proteins like cellular Bcl2 or viral E1B-19KD or acts by disrupting nuclear membrane (5, 15). Our data showing that ADP did not engage the apoptotic machinery is more in favor with the latter hypothesis that ADP mainly targets the nuclear membrane rather than modulating antiapoptotic factors to enhance oncolysis.

The role of p53 in determining the oncolytic activity of replicating Ads is somewhat controversial. After Ad infection, p53 accumulates, which is counteracted by E1B-55KD together with E4orf6, acting as E3 ubiquitin ligase, which complexes with p53, resulting in its degradation and preventing p53-dependent apoptosis. On one hand, it has been demonstrated that p53 in a complex with E1B-55KD–E4orf6 is required for efficient (rapid) oncolysis (8, 16) and that p53 overexpression (19, 44, 51) enhances the oncolytic effect of CRAds; in other studies, the presence or absence of functional p53 did not alter replication and release of Ad (26). A possible drawback in these latter studies is that tumor cell lines derived from distinct sources were compared, which does not rule out the possibility that other genetic differences apart from p53 status may influence Ad propagation. By using the isogenic cell lines H460 and H460HPV16-E6, we sought to overcome this. Our experiments with AdΔ24-based CRAd(E3+) and CRAd(E3−) did not show an effect of overexpression of the p53-sequestering HPV16-E6 protein on CRAd-induced cell killing in H460 cells, which supports the notion that p53 status does not influence CRAd-induced cell death. Our results are in agreement with reports of Harada and Bork (17) and others (18, 45), who showed comparable adenoviral production (replication and release) in cancer cells independent from p53 status, including in p53-null NSCLC cell lines (e.g., H1299) and p53-wild-type counterparts (17, 43). However, in our experiments we cannot rule out the possibility that even in H460HPV16-E6 cells only a very small portion of p53/E1B-55KD/E4orf6 is formed that is sufficient for normal oncolysis. In this scenario, p53 triggers an as-yet-unknown cell death mechanism that is distinct from classic p53-dependent apoptosis via the intrinsic (mitochondrion-mediated) route. Yet we can rule out that the E1A mutation specific to the Ad5-Δ24-based viruses plays a role in rendering CRAd(E3+)- and CRAd(E3−)-induced cell killing p53 independent, since wtAd5 induces a similar p53-independent cell killing in HPV16-E6-overexpressing H460 cells (data not shown).

Apart from addressing the role of the apoptotic machinery in CRAd-induced cell death, we also monitored a panel of morphological parameters that allows the more precise classification of the mode of cell death that can range from apoptosis to necrosis. Apoptosis, as a distinct PCD process, is mainly characterized by a combination of hallmark morphological and biochemical changes, including DNA fragmentation, chromatin condensation, cytoplasmic shrinkage, and PS externalization, which can be associated with molecular markers such as the activation of caspases (20). More recently, it has been reviewed that other types of PCD exist in which caspases are not activated despite the presence of apoptotic features, such as DNA fragmentation and chromatin condensation; this type of PCD is named apoptosis-like PCD (31). In the absence of chromatin condensation, this type of PCD is termed necrosis-like PCD, which can still be associated with apoptotic markers such as PS externalization. CRAd-induced oncolysis, regardless of the presence of the E3 region, was not associated with apoptotic DNA fragmentation caused by internucleosomal DNA cleavage (Fig. 4B). Nuclear staining revealed small aggregates (speckled staining) spread in the nucleus of CRAd-infected cells (Fig. 4A), which is difficult to assign either to aggregates of Ad DNA or to irregular chromatin condensation. By visual observation, CRAd infection seemed to increase the size of the nucleus to some extent, in contrast to the nuclear shrinkage and fragmentation observed with FasL+CHX-treated cells. Finally, Annexin V staining assays showed that CRAd-induced cell death was associated with PS externalization that could not be blocked by zVAD-fmk, in contrast to PS exposure-induced by FasL+CHX that was caspase dependent.

Regardless of the observed loss of membrane integrity in a portion of CRAd-infected cells, PS exposure was the only apoptotic marker found in this study. In Table 1, the results are summarized whereby CRAd-induced phenomena are compared to apoptosis induced by FasL+CHX treatment and four forms of cell death. It can be concluded that the detected molecular and cellular features that characterize CRAd-induced cell death rule out apoptotic or necrotic cell death and that necrosis-like PCD fits CRAd(E3+)- and CRAd(E3−)-induced cell death best, although this is inevitably somewhat arbitrary.

The found lack of involvement of the basic apoptotic machinery in mediating CRAd(E3+)- and CRAd(E3−)-induced cell death corresponds with previous data showing that wtAd type 5 (wtAd5)-induced cell kill was not associated with DNA fragmentation or the formation of apoptotic nuclei (35, 49) and also indicates that the Δ24 mutation in E1A does not alter these features. This is remarkable when considering the clear apoptotic or antiapoptotic activities of the various characterized individual adenoviral proteins (reviewed in references 6, 38, and 52). For example, E1A13S-induced apoptosis is associated with caspase-3 activation and PARP cleavage (4); Lavoie and coworkers (30) showed that E4orf4-induced p53-independent cell death was associated with loss of mitochondrial membrane potential and chromatin condensation but without caspase-3 activation or PARP cleavage. However, as we demonstrate here, when Ad proteins act in concert, the net outcome is the activation of a caspase-independent mode of cell death. This may be explained by assuming that Ad proteins that trigger caspase-independent and nonclassical apoptotic cell death dominate this process, such as the previously mentioned E4orf4 protein (7, 25). In the literature, cell death induced by replicating Ads is often referred to as apoptosis, which in light of this study is incorrect. It should be noted that different assays are used in these studies to determine apoptosis that are not suitable for making the distinction between apoptotic and nonapoptotic forms of cell death. More importantly, it should be realized that depending on the particular combination of replicating Ad vector and host cell line used, the outcome may vary. For example, the use of an Ad2-based mutant that lacks E1B-19KD (a Bcl2 homologue) expression was found to have an enhanced cytopathic effect in colon cancer HCT116 cells with a functional Bax gene when compared to non-Bax-expressing counterparts, which was interpreted as evidence for Bax contributing to Ad-induced apoptosis (34). This finding may be true for a virus with a deleted E1B-19KD gene in a Bax-positive background, but it is not relevant for a CRAd expressing the Bax-inhibiting E1B-19KD protein. Similarly, the concept that induction of cell death in Ad-infected cancer cells may enhance progeny virus release and spread has been shown for a particular combination of host cells and Ad. Mi and coworkers (39) found that in HeLa cells infected with Ad E1/E3-deleted viruses that overexpress IκB, which sensitizes cells for tumor necrosis factor α (TNF-α)-induced apoptosis, the administration of TNF-α after the virion assembly stage enhanced viral release.

For the generation of more effective CRAds, interfering with the early apoptosis-regulating properties of the Ad should be avoided when possible, to prevent adverse effects on optimal virus production. Correctly timed expression of proapoptotic genes is therefore essential and can be achieved (for example) by production from the E3 region, as reported for TNF-α (42). The expression of diffusible apoptosis-inducing proteins could also have therapeutic benefit by their ability to act at a distance from the infected cells, thereby circumventing possible barriers in the tumor mass that may hinder virus spread.

Our study indicates that deregulated apoptosis in cancer cells will likely not oppose effective CRAd-induced cell death. Their mechanism of tumor cell killing is therefore different from conventional therapies in which apoptosis activation contributes to cell death and may provide an explanation for the potency of CRAds in killing chemo-resistant tumor cells. It seems that during evolution, Ads evolved a mechanism for disrupting the host cell at the final stage of the viral cycle that does not require the activation of the basic apoptotic machinery. Currently, we are attempting to identify pathways that are involved in the activation of CRAd-induced cell death by employing microarray-based approaches. The elucidation of host cell mechanisms that affect CRAd-dependent oncolysis will help to identify CRAd-resistance mechanisms and allow the development of rationalized strategies to circumvent such hurdles.

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REFERENCES

1. **Adams, J. M., and S. Cory.** 2001. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem. Sci. 26:61–66.
2. **Alemany, R., C. Balague, and D. T. Curiel.** 2000. Replicative adenoviruses for cancer therapy. Nat. Biotechnol. 18:723–727.
3. **Bauerschmitz, G. J., J. T. Lam, A. Kanerva, K. Suzuki, D. M. Nettelbeck, L. Dmitriev, V. Krasnykh, G. V. Mikheeva, M. N. Barnes, R. D. Alvarez, P. Dall, R. Alemany, D. T. Curiel, and A. Hemminki.** 2002. Treatment ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res. 62:1266–1270.
4. **Boulakia, C. A., G. Chen, F. W. Ng, J. G. Teodoro, P. E. Branton, D. W. Nicholson, G. G. Poirier, and G. C. Shore.** 1996. Bcl-2 and adenovirus E1B 19 kDA protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase. Oncogene 12:529–535.
5. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell 79:341–351.
6. **Braithwaite, A. W., and L. A. Russell.** 2001. Induction of cell death by adenoviruses. Apoptosis 6:359–370.
7. **Branton, P. E., and D. E. Roopchand.** 2001. The role of adenovirus E4orf4 protein in viral replication and cell killing. Oncogene 20:7855–7865.
8. **Dix, B. R., S. J. O'Carroll, C. J. Myers, S. J. Edwards, and A. W. Braithwaite.** 2000. Efficient induction of cell death by adenoviruses requires binding of E1B55k and p53. Cancer Res. 60:2666–2672.
9. **Earnshaw, W. C., L. M. Martins, and S. H. Kaufmann.** 1999. Mammalian Caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68:383–424.
10. **Fadok, V. A., A. de Cathelineau, D. L. Daleke, P. M. Henson, and D. L. Bratton.** 2001. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J. Biol. Chem. 276:1071–1077.
11. **Ferreira, C. G., S. W. Span, G. J. Peters, F. A. E. Kruyt, and G. Giaccone.** 2000. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the NSCLC cell line NCI-H460. Cancer Res. 60:7133–7141.
12. **Ferrer, M., T. Izebaud, C. G. Ferreira, S. W. Span, G. Giaccone, and F. A. E. Kruyt.** 2003. Cisplatin triggers apoptotic or nonapoptotic cell death in Fanconi anemia lymphoblasts in a concentration-dependent manner. Exp. Cell Res. 286:381–395.
13. **Fueyo, J., C. Gomez-Manzano, R. Alemany, P. S. Lee, T. J. McDonnell, P. Mitlianga, Y. K. Shi, V. A. Levin, W. K. Yung, and A. P. Kyritsis.** 2000. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene 19:2–12.

14. **Ganly, I., D. Kirn, S. G. Eckhardt, G. I. Rodriguez, D. S. Soutar, R. Otto, A. G. Robertson, O. Park, M. L. Gulley, C. Heise, D. D. Von Hoff, and S. B. Kaye.** 2000. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. Clin Cancer Res. 6:798–806.
15. **Givol, L. I. Tzarfaty, J. Rossu, S. Kulong, P. P. de Silva, G. Nesioulas, J. DuHadaway, S. H. Hughes, and D. L. Ewert.** 1994. Bcl-2 expressed using a retroviral vector is localized primarily in the nuclear membrane and the endoplasmic reticulum of chicken embryo fibroblasts. Cell Growth Differ. 5:419–429.
16. **Hall, A. R., B. R. Dix, S. J. O'Carroll, and A. W. Braithwaite.** 1998. p53-dependent cell death/apoptosis is required for a productive adenovirus infection. Nat. Med. 4:1068–1072.
17. **Harada, J. N., and A. J. Berk.** 1999. p53-Independent and -dependent requirements for E1B-55K in adenovirus type 5 replication. J. Virol. 73:5333–5344.
18. **Harada, J. N., A. Shevchenko, A. Shevchenko, D. C. Pallas, and A. J. Berk.** 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J. Virol. 76:9194–9206.
19. **Haviv, Y. S., K. Takayama, J. N. Glasgow, J. L. Blackwell, M. Wang, X. Lei, and D. T. Curiel.** 2002. A model system for the design of armed replicating adenoviruses using p53 as a candidate transgene. Mol. Cancer Ther. 1:321–328.
20. **Hengartner, M. O.** 2000. The biochemistry of apoptosis. Nature 407:770–776.
21. **Hoffmann, P. R., A. M. deCathelineau, C. A. Ogden, Y. Leverrier, D. L. Bratton, D. L. Daleke, A. J. Ridley, V. A. Fadok, and P. M. Henson.** 2001. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J. Cell Biol. 155:649–660.
22. **Kataoka, M., S. Wiehle, F. Spitz, G. Schumacher, J. A. Roth, and R. J. Cristiano.** 2000. Down-regulation of bcl-2 is associated with p16INK4-mediated apoptosis in non-small cell lung cancer cells. Oncogene 19:1589–1595.
23. **Kirn, D.** 2000. Replication-selective oncolytic adenoviruses: virotherapy aimed at genetic targets in cancer. Oncogene 19:6660–6669.
24. **Kirn, D.** 2001. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther. 8:89–98.
25. **Kleinberger, T.** 2000. Induction of apoptosis by adenovirus E4orf4 protein. Apoptosis 5:211–215.
26. **Koch, P., J. Gottfield, C. Lober, U. Hobom, C. Lenz-Stoppler, J. Roth, and M. Dubbelstein.** 2001. Efficient replication of adenovirus despite the overexpression of active and nondegradable p53. Cancer Res. 61:5941–5947.
27. **Kroemer, G.** 1997. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. 3:614–620.
28. **Kruyt, F. A. E., and D. T. Curiel.** 2002. Towards a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. Hum. Gene Ther. 13:485–495.
29. **Lamfers, M. L. M., J. Grill, C. M. F. Dirven, V. W. van Beusechem, B. Geoerger, J. van den Berg, R. Alemany, J. Fueyo, D. T. Curiel, G. Vassal, H. M. Pinedo, W. P. Vandertop, and W. R. Gerritsen.** 2002. Potential of the conditionally replicative adenovirus Ad5-Δ24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy. Cancer Res. 62:5736–5742.
30. **Lavoie, J. N., M. Nguyen, R. C. Marcellus, P. E. Branton, and G. C. Shore.** 1998. E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. J. Cell Biol. 140:637–645.
31. **Leist, M., and M. Jaattela.** 2001. Four deaths and a funeral: from caspases to alternative mechanisms. Nat. Rev. Mol. Cell Biol. 2:1–10.
32. **Lichtenstein, D. L., K. Toth, K. Doronin, A. E. Tollefson, and W. S. Wold.** 2004. Functions and mechanisms of action of the adenovirus E3 proteins. Int. Rev. Immunol. 23:75–111.
33. **Lichtenstein, D. L., P. Krajcsi, D. J. Estehan, A. E. Tollefson, and W. S. M. Wold.** 2002. Adenovirus RIDβ subunit contains a tyrosine residue that is critical for RID-mediated receptor internalization and inhibition of Fas- and TRAIL-induced apoptosis. J. Virol. 76:11329–11342.
34. **Lomonosova, E., T. Subramanian, and G. Chinnadurai.** 2002. Requirement of BAX for efficient adenovirus-induced apoptosis. J. Virol. 76:11283–11290.
35. **Marcellus, R., J. Teodoro, T. Wu, D. Brough, G. Kemer, G. Shore, and P. Branton.** 1996. Adenovirus type 5 early region 4 is responsible for E1A-induced p53- independent apoptosis. J. Virol. 70:6207–6215.
36. **Martin, M. E., and A. J. Berk.** 1998. Adenovirus E1B 55K represses p53 activation in vitro. J. Virol. 72:3146–3154.
37. **McCormick, F.** 2001. Cancer gene therapy: fringe or cutting edge? Nat. Rev. Cancer 1:130–141.
38. **McNees, A. L., and L. R. Gooding.** 2002. Adenoviral inhibitors of apoptotic cell death. Virus Res. 88:87–101.
39. **Mi, J., Z. Y. Li, S. Ni, D. Steinwaerder, and A. Lieber.** 2001. Induced apoptosis supports spread of adenovirus vectors in tumors. Hum. Gene Ther. 12:1343–1352.
40. **Nemunaitis, J., F. Khuri, I. Ganly, J. Arseneau, M. Posner, E. Vokes, J. Kuhn, J. McCarty, S. Landers, A. Blackburn, L. Romel, B. Randlev, S. Haye, and D. Kirn.** 2001. Phase I trial of intratumoral administration of ONYX-015, a replication selective adenovirus, in patients with refractory head and neck cancer. J. Clin. Oncol. 19:289–298.
41. **Reed, J. C.** 1998. Bcl-2 family proteins. Oncogene 17:3225–3236.
42. **Ring, C. J. A.** 2002. Cytolytic viruses as potential anti-cancer agents. J. Gen. Virol. 83:491–502.
43. **Rothmann, T., A. Hengstermann, N. J. Whitaker, M. Scheffner, and H. zur Hausen.** 1998. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. J. Virol. 72:9470–9478.
44. **Sauthoff, H., T. Pipiya, S. Heitner, S. Chen, R. G. Norman, W. N. Rom, and J. G. Hay.** 2002. Late expression of p53 from a replicating adenovirus improves tumor cell killing and is more tumor cell specific than expression of the adenoviral death protein. Hum. Gene Ther. 13:1859–1871.
45. **Sun, X.-M., M. MacFarlane, J. Zhuang, B. B. Wolf, D. R. Green, and G. M. Cohen.** 1999. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem. 274:5053–5060.
46. **Suzuki, K., R. Alemany, M. Yamamoto, and D. T. Curiel.** 2002. The presence of the adenovirus E3 region improves the oncolytic potency of conditionally replicative adenoviruses. Clin. Cancer Res. 8:3348–3359.
47. **Suzuki, K., J. Fueyo, V. Krasnykh, P. N. Reynolds, D. T. Curiel, and R. Alemany.** 2001. A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. Clin. Cancer Res. 7:120–126.
48. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science 281:1312–1316.
49. **Tollefson, A. E., T. W. Hermiston, D. L. Lichtenstein, C. F. Colle, R. A. Tripp, T. Dimitrov, K. Toth, C. E. Wells, P. C. Doherty, and W. S. Wold.** 1998. Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature 392:726–730.
50. **Tollefson, A. E., A. Scaria, T. W. Hermiston, J. S. Ryerse, L. J. Wold, and W. S. Wold.** 1996. The adenovirus death protein (E3–11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. J. Virol. 70:2296–2306.
51. **van Beusechem, V. W., P. B. van den Doel, J. Grill, H. M. Pinedo, and W. R. Gerritsen.** 2002. Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. Cancer Res. 62:6165–6171.
52. **Wold, W. S., K. Doronin, K. Toth, M. Kuppuswamy, D. L. Lichtenstein, and A. E. Tollefson.** 1999. Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. Curr. Opin. Immunol. 11:380–386.
53. **Wu, G. S., and W. S. El-Deiry.** 1996. Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or bcl-2. Clin. Cancer Res. 2:623–633.
54. **Yang, Y. L., and X. M. Li.** 2000. The IAP family: endogenous caspase inhibitors with multiple biological activities. Cell Res. 10:169–177.

Exhibit D

BRIEF COMMUNICATION

Examination of the Therapeutic Potential of Delta-24-RGD in Brain Tumor Stem Cells: Role of Autophagic Cell Death

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The eradication of brain tumor stem cells is essential for long-term brain tumor remission after treatment. In this study, we examined the therapeutic potential of an oncolytic adenovirus, Delta-24-RGD, targeted to the abnormal p16INK4/Rb pathway in brain tumor stem cells. Four brain tumor stem cell lines from surgical glioblastoma specimens expressed high levels of adenoviral receptors and allowed for efficient viral infection, replication, and oncolysis in an Rb-dependent manner. Delta-24-RGD induced autophagic cell death, as indicated by accumulation of Atg5 and LC3-II protein and autophagic vacuoles. Treatment of xenografts derived from brain tumor stem cells with Delta-24-RGD statistically significantly improved the survival of glioma-bearing mice (means: 38.5 versus 66.3 days, difference = 27.8 days, 95% confidence interval = 19.5 to 35.9 days, $P<.001$). Analyses of treated tumors showed that Atg5 expression colocalized with viral fiber protein and delineated a wave front of autophagic cells that circumscribed areas of virally induced necrosis. Our results show for the first time that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophagy in vitro and in vivo.

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The brain tumor stem cell hypothesis proposes the existence of multipotent glioma cells of origin that are characterized by the expression of stem cell markers and by the capacity for self-renewal, multilineage differentiation, and reestablishment of tumors after transplantation (1–5). An implication of the brain tumor stem cell model is that brain tumor stem cells are resistant to radiation and chemotherapy and may therefore be responsible for tumor recurrence (6,7). Because adenoviral proteins can completely overcome the molecular machinery of the infected cell, we hypothesized that Delta-24-RGD, an oncolytic adenovirus with enhanced tropism to glioma cells and selective replication in cancer cells with an abnormal Rb pathway (8,9), may act as a potent therapeutic agent to target brain tumor stem cells and prevent them from developing resistance to other forms of therapy. However, adenoviral receptor expression, infectibility, the susceptibility to adenoviral replication, as well as the characteristics of adenovirus-mediated cell death have not previously been examined in cancer stem cells.

In this study, we isolated neurosphere-forming cells from four fresh surgical specimens of glioblastoma multiforme (3) (Fig. 1, A). These cells exhibited the in vitro stem cell characteristics of extensive self-renewal (more than five passages in culture) and the ability to differentiate to neurons and astrocytes (Supplementary Fig. 1, available online). Flow cytometric analyses showed that 20%–80% of the cells expressed the neural stem cell protein CD133, which was recently identified as a potential brain tumor stem cell marker in brain cancer (1) and in other solid tumors (10,11) (Fig. 1, A). When clonally derived, these cells initiated new tumors when transplanted into the basal ganglia of immunodeficient mice (Supplementary Fig. 2, available online). Because Delta-24-RGD is targeted to Rb-deficient cells (8,9), we examined the levels of Rb and p16INK4a proteins, whose expression is mutually exclusive in glioblastoma multiforme (12,13), in brain tumor stem cells. As reported for glioblastoma multiforme, immunoblotting analyses showed that the expression of either Rb or p16 protein was absent in these cell lines (Fig. 1, B). Next, using flow cytometric analysis, we demonstrated that the cell lines expressed high levels (>50% positive cells) of coxsackie–adenovirus receptor, the main adenoviral receptor required for virus attachment (14), and/or Arg-Gly-Asp (RGD)–recognizing integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ for virus internalization (15) (Fig. 1, C). Consequently, the four glioma stem cell lines were susceptible to adenoviral infection (Supplementary Fig. 3, A, available online). Accordingly, treatment of the cell lines with Delta-24-RGD resulted in a drastic reduction in cell viability, and at 6 days after viral infection, the dose inducing 50% cell death (ID50) was less than 2 pfu/cell in the majority of the cell lines, as assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Fig. 1, D). As expected, pretreatment with Rb protein resulted in rescue of the viability of Delta-24-RGD–infected cells that was due

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to the restriction of an efficient replication phenotype (Fig. 1, E and F; Supplementary Fig. 3, B, available online).

The pathways involved in adenovirus-mediated cell death remain unclear. Here, we showed that Delta-24-RGD induced the formation of acidic vesicular organelles in the four cell lines ($P<.001$) (Fig. 2, A; Supplementary Fig. 4, A, available online). The Delta-24-RGD–induced acidic vesicular organelles were further confirmed by the dramatic change in the ratio of the cytosolic LC3-I to membrane-bound LC3-II (Supplementary Fig. 4, B, available online), a modification that is essential for the formation of autophagosomes (16), in the treated cells. These data were consistent with a previous report showing adenovirus-induced autophagy in two established glioma cell lines (17). The observation of increased levels of biochemical markers and cellular acidic vesicular organelles was strongly supported by the direct demonstration of cytoplasmic autophagic vacuoles in cells treated with Delta-24-RGD using electron microscopy (Fig. 2, B). To examine the activation of proautophagic signaling by Delta-24-RGD infection, we first examined the protein levels of beclin1, a type III PI3 kinase–interacting protein that participates in the induction of autophagy (18). Western blot analyses showed no differences in the levels of beclin1 in treated versus untreated cells (Supplementary Fig. 4, B, available online). We next analyzed the protein levels of Atg5, a key molecule in the conversion of LC3-I to -II and therefore required for autophagosome formation and autophagic cell death (19), during the Delta-24-RGD replication cycle. We observed a remarkable induction of endogenous Atg5 expression that was noticeable by 48 hours after treatment and was increased by six- to eightfold 72 hours after the treatment, the latest time point examined (Fig. 2, C; Supplementary Fig. 4, B, available online). The timing of Atg5 expression suggested that activation might link Delta-24-RGD–mediated cell lysis to autophagic cell death.

We next examined the anti-tumor efficacy of Delta-24-RGD in intracranial xenografts that were derived from MDNSC11 cells in athymic mice. The mean survival time of control-treated MDNSC11-glioma–bearing mice was 38.5 days (95% confidence interval [CI] = 35.6 to 41.4 days), in contrast to a mean survival time of 66.3 days (95% CI = 55.2 to 77.3 days, the largest observed analysis time is censored, the mean is underestimated) in mice treated with Delta-24-RGD (difference = 27.8 days, 95% CI = 19.5 to 35.9 days; $P<.001$) (Fig. 2, D) with two of eight (25%) mice remaining alive without noticeable neurologic deficits until they were killed at day 92 (Fig. 2, D). Microscopic examination of the brain tissues of control mice with MDNSC11 xenografts revealed highly infiltrative tumors that recapitulated the histopathology of glioblastomas including hypercellularity, hypervascularity, and necrotic areas surrounded by cells in a pseudopalisading distribution (Supplementary Fig. 2, available online). Immunohistochemical staining of the tumor from the Delta-24-RGD–treated group revealed the expression of E1A and hexon indicating efficient adenoviral infection and replication in vivo (Supplementary Fig. 5, available online). Importantly, immunofluorescence analyses identified high levels of expression of the proautophagic protein Atg5 (Fig. 2, E). Atg5 colocalized with fiber protein and displayed a pattern of expression that clearly defined a tumor zone immediately adjacent to the necrosis area (Fig. 2, E). No other area in the tumor or any area of untreated tumors was positive for Atg5 (data not shown). Therefore, Atg5 appears to be useful to identify the adenoviral wave front of spread and may be used as a cellular indicator of viral activity and a surrogate marker of the anti-glioma effect.

In summary, our results show for the first time, to our knowledge, that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophaghy in vitro and in vivo. Our data are important because brain tumor stem cells are the driving force sustaining tumor growth (1,3), and therefore developing therapies to target the brain tumor stem cells should be a more effective strategy than conventional treatments. We reported previously that Delta-24-RGD displays efficacious anti-glioma activity in the models based on established malignant glioma cell lines (9). Together, our previous study and this study indicate that Delta-24-RGD efficiently eliminates both brain tumor stem cells and tumor mass cell populations in gliomas. An alternative theory to the cancer stem cell model hypothesizes that mutant dedifferentiated astrocytes are responsible for the emergence and phenotype of high-grade gliomas (20). Nevertheless, despite the origin of these tumor-initiating cells, the resulting phenotype of the cancer cell is probably similar (20), and the universal disruption of the Rb pathway renders them susceptible to Delta-24-RGD (Fig. 1, B; 8,9,20). Furthermore, the in vivo assessment of adenovirus-induced autophagy may be a useful way to monitor oncolytic adenovirus efficacy in future clinical trials.

CONTEXT AND CAVEATS

Prior knowledge

To achieve long-term remission after treatment for brain cancer, it is necessary to eradicate brain tumor stem cells that are resistant to radiation and chemotherapy.

Study design

The therapeutic potential of oncolytic adenovirus Delta-24-RGD targeted to brain tumor cells was tested in vitro using cell lines with stem cell properties that were derived from glioblastoma multiforme tumors and in vivo using a mouse xenograft tumor model.

Contribution

The four cell lines were efficiently infected with an oncolytic adenovirus Delta-24-RGD, which induced autophagic cell death. Mice carrying xenograft tumors that were derived from one of the cell lines survived longer after treatment with Delta-24-RGD than with an inactivated form of the virus.

Implications

Adenovirus-mediated autophagic cell death can be induced in brain tumor cells with properties of stem cells.

Limitations

It is unclear how similar the cell lines developed in this study are to the brain tumor stem cells that exist in human brain cancer or whether the oncolytic adenovirus developed in this study would be efficacious and safe in humans.

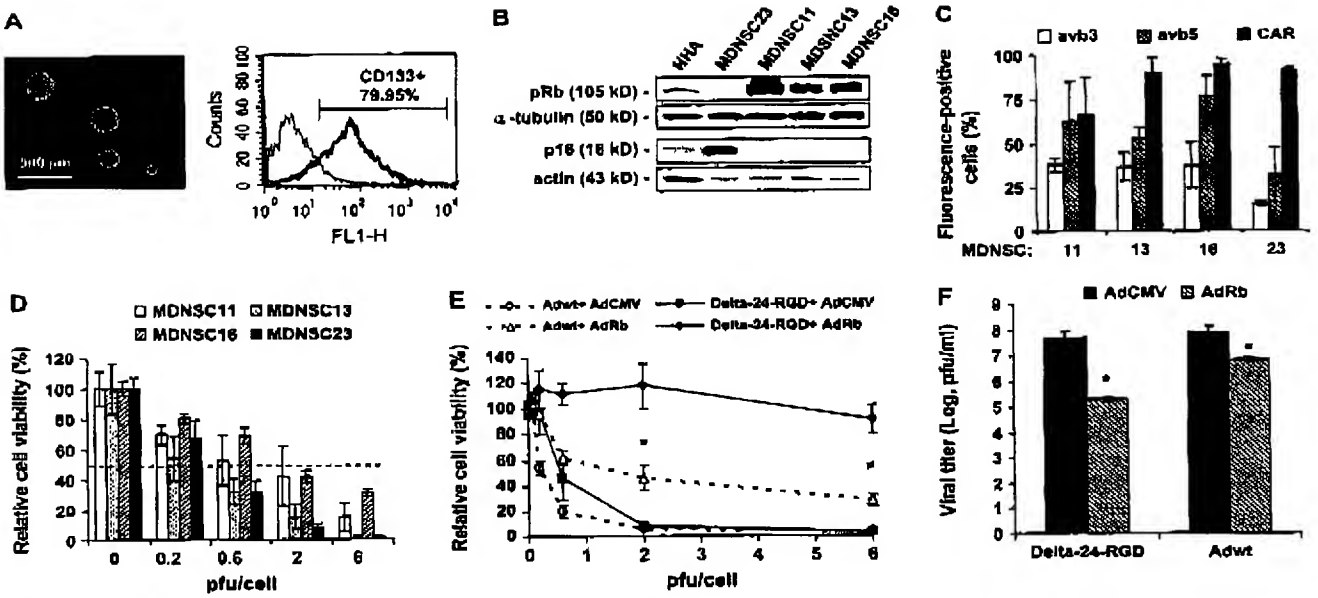

Fig. 1. Delta-24-RGD–mediated cytotoxicity in cell lines with properties of brain tumor stem cells. **A)** Neurosphere cultures were established from acute cell dissociation of four human glioblastoma multiforme surgical specimens (2,3) and maintained in Dulbecco's modified Eagle medium/F12 medium supplemented with B27 (Invitrogen, Carlsbad, CA), epidermal growth factor, and basic fibroblast growth factor (20 ng/mL each, Sigma–Aldrich, St Louis, MO) according to the procedures described elsewhere (2,3). The study was approved by the Institutional Review Board at University of Texas M. D. Anderson Cancer Center. Written informed consent was obtained from every patient. Shown are results from one representative of four experiments. **Left**, representative image of formation of typical neurospheres in MDNSC16 cultures, phase-bright microphotograph. **Right**, CD133 expression in MDNSC16 cells. The cells were dissociated by exposure to accutase solution (Sigma–Aldrich) and pipetting and stained with monoclonal antibody specific for CD133 (1:10 dilution; Miltenyi Biotec, Auburn, CA) and then with fluorescein isothiocyanate (FITC)–labeled secondary antibody (anti-mouse IgG, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) according to the instructions provided by the manufacturer. After staining, the cells were analyzed by flow cytometry for cell surface expression of CD133 with FacsCalibur (Becton Dickinson, San Jose, CA). **B)** Expression of Rb protein (pRb) and p16INK4a (p16) protein in the cell lines. Normal human astrocytes (NHAs) were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, MD) and maintained in astrocyte growth medium prepared from an AGM-Astrocyte Medium BulletKit (Cambrex Bio Science Walkersville, Inc) as instructed by the manufacturer. Western blots were performed as described previously (9). Briefly, equal amount of proteins from whole-cell lysates were separated by 8% (pRb) or 15% (p16) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and the membranes were probed with rabbit polyclonal anti-pRb (1:200 dilution), goat polyclonal anti-p16INK4a (1:100 dilution), goat polyclonal anti-actin (1:1000 dilution) (Santa Cruz Biotechnology), and mouse monoclonal anti–α-tubulin (1:4000 dilution; Sigma–Aldrich). The protein–antibody complexes were visualized using the enhanced chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). α-tubulin and actin were used as loading controls. One representative experiment of two is shown. **C)** Flow cytometric analysis of αvβ3, αvβ5 integrins and coxsackie–adenovirus receptor (CAR) expression in the cell lines. The analysis was performed as described previously (9). Dissociated cells (5×10^5) were stained with mouse monoclonal anti-αvβ3 (1:200 dilution; Chemicon International, Inc, Temecula, CA), anti-αvβ5 (1:400 dilution; Chemicon International, Inc), or anti-CAR (1:2000 dilution; Upstate Biotechnology, Lake Placid, NY) and then with FITC-labeled secondary antibody goat anti-mouse IgG (1:100 dilution, Santa Cruz Biotechnology). The stained cells were analyzed with flow cytometry for cell surface expression of viral receptors using a FacsCalibur flow cytometer (Becton Dickinson). Three independent experiments were performed. Data are shown as the mean (and 95% confidence intervals [CIs]) percentage of cells expressing the receptors. **D)** Cytotoxicity induced by Delta-24-RGD in the cell lines. The cells were dissociated and plated in 96-well plates at 2×10^4 cells per well and were immediately infected with Delta-24-RGD at the indicated doses. Six days after infection, cells were dissociated with accutase solution (400–600 U/mL, Sigma–Aldrich) and by pipetting before cell viability was assessed using the MTS assay according to the manufacturer's instructions (Promega Life Science, Madison, MI). The experiments were performed once in triplicate. Data are shown as mean (and 95% CIs) percentage of viable cells relative to that of mock-treated cells (equal to 100%). **E)** Effect of pRb restoration on the Delta-24-RGD–mediated cytotoxicity in MDNSC23 cells. The cells were infected with replication-deficient adenovirus expressing Rb protein, AdRb, or the control E1A-deleted virus AdCMV at 150 pfu/cell, as previously reported (9). Forty-eight hours later, cells were plated (2×10^4 per well) in 96-well plates and infected with Delta-24-RGD or wild-type adenovirus (Adwt) at the indicated doses. Six days later, cell viability was determined using the MTS assay as described in (**D**). The experiments were performed once in triplicate. Data are shown as the mean (and 95% CIs) percentage of viable cells relative to that of mock-treated cells (equal to 100%). At viral dosages of 2 and 6 pfu/cell, the Rb-mediated rescue of the cytotoxicity induced by Delta-24-RGD treatment is statistically significantly greater than the Rb-mediated rescue in cells treated with Adwt (*$P = .01$, #$P = .03$, two-tailed Student's *t*-test). **F)** Effect of pRb restoration on the replication of Delta-24-RGD in MDNSC23. Cells were infected with AdRb or control virus AdCMV at 150 pfu/cell, as described in (**E**). Forty-eight hours later, the cells were infected with Delta-24-RGD or Adwt at 1 pfu/cell. The titers of the viral progenies were determined 72 hours after viral infection using the tissue culture infection dose$_{50}$ method, as described previously (9). Shown are the titers of viral progenies from 1×10^5 cells in 1 mL infected with the indicated viruses. The experiments were performed once in triplicate. Means (and 95% CIs) are shown. The Rb-mediated restriction on Delta-24-RGD replication is statistically significantly greater than that on Adwt in MDNSC23 cells (*$P = .009$, two-tailed Student's *t*-test).

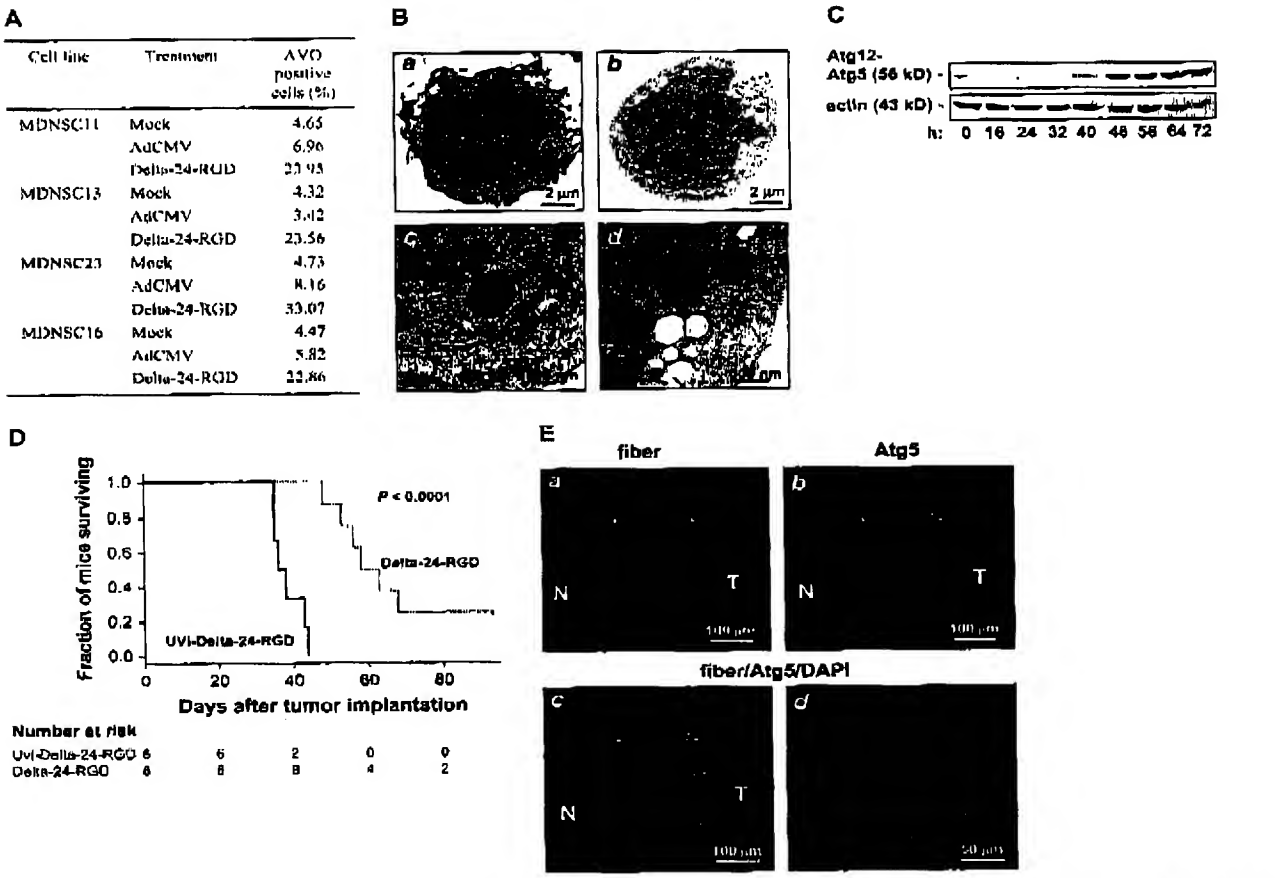

Fig. 2. Examination of the in vitro and in vivo Delta-24-RGD–induced autophagy. **A)** Induction of acidic vesicular organelles (AVOs) by Delta-24-RGD in cell lines with properties of brain tumor stem cells. Spheroids were dissociated and immediately infected with the indicated virus at 10 pfu/cell. Seventy-two hours later, cells were dissociated 2 hours before acridine orange (1μg/mL; Sigma–Aldrich) was added to the medium for 15 minutes. Percentage of cells presenting the formation of AVOs were quantified by flow cytometry as previously described (17). AdCMV was used as a control for the viral infection. Note that Delta-24-RGD induced a statistically significant increase of the percentage of AVO-positive cells in all cell lines tested compared with AdCMV (means: 26.8% versus 6.1%, difference = 19.7%, 95% confidence interval = 15.0% to 24.3%, $P<.001$, randomized block two-factor analysis of variance). **B)** Representative electron micrographs showing the ultrastructure of the mock-infected (a) and Delta-24-RGD–infected (b) MDNSC11 cells. Note the vacuoles in the virus-infected cells but not in the untreated cells. Close-ups of Delta-24-RGD–infected cell illustrated in b show the cluster of the progenies of Delta-24-RGD (**white arrow**) in the nucleus (c) and complex autophagic multivacuolar bodies in the cytoplasm (d). Representative images from 20 cells are shown. **C)** Kinetics of Atg12-Atg5 protein expression during Delta-24-RGD infection. MDNSC11 cells were collected at the indicated time points after Delta-24-RGD infection, and equal amounts of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membranes, and the membranes were probed with rabbit polyclonal anti-Atg6 (1:2000 dilution; a kind gift from Dr N. Mizushima, National Institute for Basic Biology, Japan) and goat polyclonal anti-actin antibodies. The protein–antibody complexes were visualized as described (in Fig. 1, B). Actin was used as a loading control. Data from one representative experiment of two are shown. **D)** Kaplan–Meier curves of overall survival of Delta-24-RGD–treated (n = 8) and UV-inactivated (UVI) Delta-24-RGD–treated (n = 6) athymic mice with MDNSC11 intracranial xenografts. Cells (1.0×10^6) were grafted into the caudate nucleus of the athymic mice using a guide-screw system as previously described (9). On days 7, 9, and 11 after tumor cell implantation, mice were intratumorally injected with 10 μL of Delta-24-RGD or UVI-Delta-24-RGD (2.5×10^9 pfu). Survival in the two treatment groups was compared using the log-rank test (two-sided). All mouse studies were performed in the veterinary facilities of University of Texas M. D. Anderson Cancer Center in accordance with institutional, state, and federal laws and ethics guidelines for experimental animal care. **E)** Viral replication and autophagy in vivo. Immunofluorescence analysis of viral fiber and Atg5 proteins expression in the brain of a mouse from the study represented in (D) that was treated with Delta-24-RGD and died 58 days after implantation. The paraffin-embedded section of the mouse brain was double immunostained with mouse monoclonal antibodies specific for adenoviral fiber protein (4D2, 1:500 dilution; Lab Vision, Fremont, CA) (a) or rabbit polyclonal Atg5 (1:200 dilution) (b) and then with Texas Red (fiber)– or Alexa Fluor 488 (Atg5)–conjugated secondary antibodies (1:500 dilution; Molecular Probes, Inc. Eugene, OR). Fluorescence staining for fiber and Atg5 were merged and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining was used to visualize the cell nuclei (c). Expression of both proteins was positive (**double-headed arrow**) within the tumor (T) surrounding necrotic areas (N). Note, in a close-up of (c), that the viral and the cellular protein are localized in the same cells, around the cells that exhibit virally induced necrosis (d). These are the representative images from the brains of two mice treated with Delta-24-RGD.

References

(1) Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401.

(2) Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391–403.

(3) Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004;64:7011–21.

(4) Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia 2002;39: 193–206.

(5) Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 2003;100:15178–83.

(6) Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005; 5:275–84.

(7) Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006; 444:756–60.

(8) Fueyo J, Gomez-Manzano C, Alemany R, Lee PS, McDonnell TJ, Mitlianga P, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene 2000;19:2–12.

(9) Fueyo J, Alemany R, Gomez-Manzano C, Fuller GN, Khan A, Conrad CA, et al. Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. J Natl Cancer Inst 2003;95:652–60.

(10) Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.

(11) O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–10.

(12) Fueyo J, Gomez-Manzano C, Bruner JM, Saito Y, Zhang B, Zhang W, et al. Hypermethylation of the CpG island of p16/CDKN2 correlates with gene inactivation in gliomas. Oncogene 1996;13:1615–9.

(13) Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A, Louis DN. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Res 1996;56:150–3.

(14) Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997;275:1320–3.

(15) Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993; 73:309–19.

(16) Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19:5720–8.

(17) Ito H, Aoki H, Kuhnel F, Kondo Y, Kubicka S, Wirth T, et al. Autophagic cell death of malignant glioma cells induced by a conditionally replicating adenovirus. J Natl Cancer Inst 2006;98:625–36.

(18) Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999;402:672–6.

(19) Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001;152:657–68.

(20) Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, et al. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer Cell 2002;1: 269–77.

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Exhibit E

Cancer Gene Therapy (2002) 9, 1022–1035

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Review

Armed therapeutic viruses: Strategies and challenges to arming oncolytic viruses with therapeutic genes

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Oncolytic viruses are attractive therapeutics for cancer because they selectively amplify, through replication and spread, the input dose of virus in the target tumor. To date, clinical trials have demonstrated marked safety but have not realized their theoretical efficacy potential. In this review, we consider the potential of armed therapeutic viruses, whose lytic potential is enhanced by genetically engineered therapeutic transgene expression from the virus, as potential vehicles to increase the potency of these agents. Several classes of therapeutic genes are outlined, and potential synergies and hurdles to their delivery from replicating viruses are discussed.

Cancer Gene Therapy (2002) **9**, 1022–1035 doi:10.1038/sj.cgt.7700542

Keywords: oncolytic virus; armed therapeutic virus; gene therapy; cancer

Tumor-selective, replication-competent oncolytic viruses offer several unique features as cancer therapeutics. First, the input dose is amplified in a tumor-dependent fashion. Consequently, even if only a small proportion of the input dose infects some of the target tumor cells, this infective dose should be capable of replicating in and eliminating neoplastic cells, using successive waves of replication and lysis until the tumor mass is completely destroyed. Importantly, these tumor-selective replication competent viruses spare normal tissue. Because replication-selective oncolytic viruses do not replicate efficiently in normal cells, the associated toxicities should be low. This property will become critical for systemic viral delivery to treat metastatic disease. Low toxicity creates an opportunity for the investigator to increase the dose of the therapeutic virus to overcome losses associated with nonspecific uptake or neutralization due to specific (e.g., antibodies) and nonspecific (e.g., albumin) factors. With their capacity to be carried passively throughout the body via the blood or lymph circulatory systems, these agents should be able to reach, infect, and similarly eliminate all metastatic lesions. These replication-competent, tumor-specific oncolytic viruses offer hope in the daunting field of cancer therapy.

A number of replication-competent, tumor-selective oncolytic viruses have entered the clinic. Clinical experiences show that these agents are safe, but are not potent enough as monotherapies to effect complete tumor regressions or to generate sustained clinical responses. Insufficient or inefficient infection of tumor cells is generally observed. Three strategies are being pursued to overcome this weakness. One is to create less attenuated (more potent) viruses either through use of alternative viruses or by employing alternative, less attenuating, mechanisms for restricting replication to tumor cells.[1–3] The second is to employ additional cytotoxic mechanisms, beyond the direct lytic functions of the virus, by arming these viruses with therapeutic genes.[4] Particularly attractive in this context are those cytotoxic mechanisms with potent bystander effects capable of eliminating tumor cells that the virus cannot reach. And the third is to combine the oncolytic viral therapy with the more traditional radiotherapy and/or chemotherapy, with which virotherapies often synergize.[5]

This review will summarize current clinical results with replication-selective oncolytic viruses (Table 1). We will examine gene therapy strategies using nonreplicating viral vectors, as these inform current strategies for improving oncolytic therapies. Particular focus will be given to strategies for arming oncolytic viruses with therapeutic genes capable of eliciting antitumor immune function, inhibition of tumor neovascularization, or prodrug activation. Through synergistic combination of several cytotoxic modalities (viral lysis, immune or antiangiogenic function, surgery and/or chemo- and radiotherapy), therapies capable of eradicating tumors may be generated.

Oncolytic viruses

Since the early 1900s, reports of tumor regression correlating with either viral vaccination or infection have peaked interest in the oncolytic potential of viruses. The first clinical trial of replicating viruses (using wild-type adenoviruses) was done in 1956.[6] There are suggestions of efficacy in the results of that trial, but lack of understanding of both the disease and

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Table 1 Oncolytic viruses

Viral agent	Genetic alteration	Target tissue or cell pathway	Therapeutic gene	Indication	Stage of clinical development	Reference
Adenoviruses						
ONYX-015 (dl1520)	E1B–55 kDa deletion	p53	–	Head and neck Ovarian cancer Colorectal cancer Pancreatic cancer Hepatocellular Carcinoma	Phase III Phase I Phases I–II Phase I Phase I Phases I–II	[3,6,104–108]
Ad5-CD/TKrep	E1B–55 kDa deletion	p53	*CD/TK fusion*	Prostate cancer	Phase I	[30,109]
Ad.TK^{rc}(II)	E1B–55 kDa deletion	p53	*TK*	Colon cancer	–	[110]
dl922–947	E1A mutation	Rb pathway	–	Solid tumors	–	[111]
Δ24	E1A mutation	Rb pathway	–	Solid tumors	–	[112,113]
E1Adl01/07	E1A mutation	Proliferating cells	–	Solid tumors	–	[114]
KD1, KD3	E1A mutation	Proliferating cells, immunoprivileged state of tumor	–	Solid tumors	–	[115]
KD1-SPB	E1A mutation/promoter driving E4	Proliferating cells, immunoprivileged state of tumor	–	Lung cancer	–	[116]
CV706	PSA promoter-driven E1A	Prostate	–	Prostate cancer	Phases I–II	[117,118]
CV787	Probasin-driven E1A and PSA-driven E1B	Prostate	–	Prostate cancer	Phases I–II	[119]
vcF11	Tcf4-driven E1A and E4	Colon	–	Colon cancer	–	[120]
ONYX-411	E2F-driven E1A and E4	Rb pathway	–	Solid tumors	–	[1]
AvE1a04i	α-Fetoprotein–driven E1A	Liver	–	Hepatocellular carcinoma	–	[121]
ONYX-304	E3–gp19 kDa deletion	Immunoprivileged state of tumor	*CD*	Solid tumors	–	[89]
ONYX-323	E3-gp19 kDa deletion	Immunoprivileged state of tumor	*TNF*	Solid tumors	–	[89]
IG.Ad5E1(+), E3TK	E3-gp19 kDa deletion	Immunoprivileged state of tumor	*TK*	Solid tumors	–	[96]
AdTyrΔ24, AdTyrΔ2Δ24	Tyrosinase promoter-driven mutant E1A	Melanoma	–	Melanoma	–	[122]

Ad.Flk-1, Ad.Flk-Endo	Flk promoter-driven; E1A±endogilin promoter-driven E1B	Dividing endothelium	–	Solid tumors	–	[53]
01/PEME	p53-responsive promoter-driven E2F antagonist to control E1A and E2A expression	p53	–	Solid tumors	–	[123]
AdE2F-1CRc	E2F promoter-driven E1A	Proliferating cells	–	Solid tumors	–	[124]
AdAFPep/Rep	AFP promoter-driving E1A 13S, E1B–55 kDa deleted	p53	–	Hapatocellular carcinoma	–	[125]
Adl118	E1B deleted	p53	–	Breast cancer	–	[126]
Ad.DF3-E1	DF3/MUC1 promoter-driven E1A	MUC1-positive human carcinomas	*TNF*	Breast cancer	–	[86]
Adp53rc	ADP deletion	Unclear	*p53*	Solid tumors	–	[22]
HSV–derived viruses						
G207	γ34.5 and ICP6 deletion	Proliferating cells, IFN	–	Malignant glioma	Phases I–II	[8,127]
1716	γ34.5 deletion	Proliferating cells, IFN	–	Malignant glioma	Phase I	[9,128]
NV1020 (R7020)	γ34.5 deletion	Proliferating cells, IFN	–	Solid tumors	Phase I	[129]
3616UB	Uracil DNA glycosylase and γ34.5 deletion	Proliferating cells, IFN	–	Solid tumors	–	[130]
M002	γ34.5 deletion	Proliferating cells, IFN	*IL-12*	Solid tumors	–	[131]
Fu-10	γ34.5 and ICP6 deletion, selected for syncytial formation	Proliferating cells, IFN	–	Solid tumors	–	[132]
rRp450	ICP6 deleted	Proliferating cells	*CYP2B1*	Colon cancer	–	[133,134]
hrR3	ICP6 deleted	Proliferating cells	–	Solid tumors	–	[135,136]
dvB7Ig/G207	γ34.5 and ICP6 deletion	Proliferating cells, IFN	Soluble *B7-1*	Solid tumors	–	[137]
G92A	Albumin promoter-driven ICP4	Liver	–	Hepatocellular carcinoma	–	[138]
G47Δ	γ34.5, ICP6, and ICP47 deleted	Proliferating cells, IFN, immunoprivileged state of tumor	–	Solid tumors	–	[137]
dlsp+K	TK deleted	Proliferating cells	–	Solid tumors	–	[139]
R8306	γ34.5 deleted	Proliferating cells, IFN	*IL-4*	Solid tumors	–	[85]
Myb34.5	ICP6 deleted, B-myb promoter driving γ34.5	Proliferating cells, IFN	–	Solid tumors	–	[140]

(continued on next page)

Table 1 (*continued*)

Viral agent	Genetic alteration	Target tissue or cell pathway	Therapeutic gene	Indication	Stage of clinical development	Reference
NV1034	γ34.5 deleted	Proliferating cells, IFN	*GM-CSF*	Solid tumors	–	[87]
NV1042	γ34.5 deleted	Proliferating cells, IFN	*IL-12*	Solid tumors	–	[87]
HSV1yCD	ICP6 deleted	Proliferating cells	*CD*	Solid tumors	–	[141]
	γ34.5 deleted	Proliferating cells, IFN	–	Solid tumors	–	[142]
Newcastle disease virus						
PV701	Passage attenuated	IFN	–	Solid tumors	Phases I–II	[10]
Vaccinia						
Various names	TK deleted	Proliferating cells	–	Solid tumors	–	[143–146]
Vaccinia/GM-CSF RV	TK deleted	Proliferating cells	*GM-CSF*	Melanoma	Phase I	[147]
VvEMAP	TK deleted	Proliferating cells	*EMAP-II*	Melanoma	–	[148]
VV-IL-2	TK deleted	Proliferating cells	*IL-2*	Malignant mesothelioma	Phase I	[149]
VMPPNP	TK deleted	Proliferating cells	*PNP*	Solid tumors	–	[144]
VvCD	TK deleted	Proliferating cells	*CD*	Colon cancer	–	[148]
Various names	TK deleted	Proliferating cells	*B7-1, ICAM-1, LFA-3* alone and altogether in a single agent	Solid tumors		[150] and references therein
VvDD-GFP	TK and VGF deleted	Proliferating cells	–	Solid tumors		[151]
Various names	TK deleted	Proliferating cells	*GM-CSF, IFN-γ, TNFα, IL-1β*, alone and combined	Solid tumors		[145,146]
Reovirus						
Type III	None	IFN	–	Solid tumors	Phase I	[102,152]
Polio virus						
PV1 (RIPO)	IRES substitution	Malignant glioma	–	Solid tumors	–	[153]
Vesicular stomatitis virus						
Indiana strain	None	IFN	–	Solid tumors	–	[103,154]
Measles virus						
MV-Edm	Passage attenuated	IFN	–	Ovarian cancer	Phase I	[155,156]

the viral therapeutic agent prevented the development of this oncolytic system. Since then, several replication-selective oncolytic viruses have been tested extensively in the clinic: ONYX-015, Ad5-CD/TKrep, and CV787 and CV706 (all Ad5-derived); 1716 and G207 (both HSV-1–derived); and PV701 and MTH-68/H (Newcastle disease viruses).[7–14] In the clinical setting, these viruses have been administered by many routes: intratumoral, intravenous, intracranial, and intraperitoneal. Safety has been consistently high, toxicity very low, and only in the case of PV701 has a maximum tolerated dose (MTD) been established.[12] Hundreds of courses of virotherapy have been given with no adverse events attributable to the virotherapy itself. For instance, one patient has received over 30 courses of PV701.[12] Especially encouraging is the observation that where preexisting and acquired neutralizing antibodies to these oncolytic agents have been demonstrated, there has been no correlation between these titers and efficacy.[5,12,15]

To date, however, the clinical experience of these single-agent therapies has fallen short of their theoretical promise. In a few cases, full and relatively durable (up to 31 months) cures have been achieved.[12] However, most patients have not experienced measurable regressions. With ONYX-015, the replication-selective oncolytic virus that has been most extensively tested and optimized in the clinic, only 14% of patients showed objective responses due to treatment.[9] Additionally, maintenance of regressions required continuous dosing. Once virotherapy was discontinued, patients suffered early relapses. However, the patients in these trials (mostly Phase 1) had failed multiple previous treatments, including surgery, chemo-, and radiotherapies and, consequently, it is commendable and encouraging in these early trials that even 14% of this group responded. However, the distance between the promise of complete and durable tumor eradication by the oncolytic virus, and the results outlined point to a need for improved virotherapy if this is to become a viable treatment for cancer patients in the clinic. In this review, we will examine strategies to increase the efficacy of oncolytic virotherapy through the addition of therapeutic transgenes to generate what have been termed "armed therapeutic viruses",[4] focusing on therapeutic genes currently being used in nonreplicating and replicating viral-based cancer gene therapies and the methods to control their expression in the context of the replicating virus. The potential synergies and challenges these therapeutic agents may hold for a replication-dependent viral-based therapy will also be discussed.

Armed therapeutic viruses

The experience of the oncologist in the clinic and our clearer understanding of the complexity and plasticity of human solid tumors dictate that combination therapies will need to be employed to generate effective, durable responses for the cancer patient. Armed therapeutic viruses that couple the lytic capability of the virus with the capacity to deliver therapeutic factors (armed therapeutic viruses[4]) to more effectively attack the complexity associated with human tumors,[16] then, is a natural evolution of the oncolytic virus-based therapy. This approach takes advantage of the viruses' ability to selectively replicate and spread in the tumor mass to safely and efficiently deliver therapeutic genes to target tissues where the therapeutic gene products can accumulate at times and to levels that afford maximal patient benefit. Choosing the appropriate gene(s) with which to arm the oncolytic virus to enable it to arrest or eradicate the highly plastic, rapidly evolving tumor is a major question that has no simple answers. As a starting point it will be important to consider the potential interactions of the therapeutic factors with the viral-based therapies as a starting point. Several classes of gene therapy–based therapeutics have been traditionally associated with nonreplicating viral-based gene delivery vehicles (antioncogenes, tumor suppressor genes, prodrug-converting enzymes, antiangiogenic, and immunology-based gene therapies). We will briefly review these "genetic payloads," examining the different factors as candidates for delivery from the oncolytic virus and potential issues surrounding each.

Tumor suppressors and antioncogenes as therapeutic transgenes

The study of cancer molecular biology has led to the discovery of a large variety of oncogenes and tumor suppressors whose aberrant expression or function causes oncogenic transformation. Numerous preclinical studies using replication-defective viruses have shown that restoration of tumor suppressor function, or inhibition of oncogene function, slows tumor growth and/or leads to apoptosis or cancer cell death. The theoretical bases for these virotherapies have been reviewed recently in several articles. One disadvantage of virotherapy strategies based on oncogene inhibition stems from the fact that only infected cells in which the transgene is expressed are killed. No bystander effects due to oncogene inhibition have been observed, i.e., uninfected tumor cells are not killed. Current virotherapy vectors are not efficient enough to insure infection of even the majority — much less all — of the tumor cells, even after intratumoral injection.

A second disadvantage of using oncogene inhibitors or tumor suppressors to arm replication-competent oncolytic viruses is that the action of the inhibitors and suppressors, while toxic to the target tumor cell, is also likely to attenuate viral replication.[20] It may be that restraining expression of the oncogene inhibitor or tumor suppressor therapeutic transgene until late in the viral life cycle, when viral replication is essentially complete, would avoid this counterproductive conflict.[4]

A third possible interference between this class of therapeutic transgenes and the replicating vector encoding them stems from the fact that tumor suppressors and oncogene inhibitors generally affect a number of pathways in the cell, any of which may compromise the engineered or endogenous tumor selectivity mechanism of the oncolytic virus or the viruses' ability to replicate in the target tumor cell. The former would be detrimental to safety, and the latter to efficacy.

However, therapies based on expression of tumor suppressors may be more effective than has been predicted based on their known mechanisms of action. For example, *p53* gene transfer studies have unexpectedly demonstrated that the expression of p53 can trigger a number of events to generate beneficial bystander effects,[21–23] any or all of which may synergize with the viral infection. More recently, investigators engineered the *p53* gene into a replicating adenovirus from which it was expressed to high levels at late times postinfection. Surprisingly, this virus demonstrated enhanced preferential lysis of tumor cells to the exclusion of normal cells.[24] The ability of antioncogenes to synergize with the viral infection remains to be tested.

Prodrug therapies

The efficacy of traditional chemotherapies has been hampered by dose-limiting toxicities to normal cells. Prodrug therapies seek to reduce this toxicity by selectively generating the chemotherapeutic agent at the target tumor site. Such prodrug-based cancer therapies have two basic components: an inactive, nontoxic prodrug and a prodrug-activating enzyme (for a recent list, please see Ref. [15]). In this anticancer strategy, the prodrug can (ideally) be delivered systemically at high doses. The prodrug only becomes cytotoxic when activated by the appropriate enzyme. If the activating enzyme is expressed exclusively in tumor cells, then the prodrug will be activated, or become cytotoxic, only at the site of the target cancer cell. Ideally, once activated, the chemotherapeutic drug leaves the cell in an activated cytotoxic form to kill surrounding tumor cells (local bystander effect). Such bystander effects are particularly important to compensate for the inefficient infection and transduction of tumor cells by currently available vectors. Preclinical demonstrations of bystander effects using various prodrug and activating enzyme combinations have been published. In these studies, tumors composed of as few as 10% of prodrug-expressing cells were fully eradicated, whereas control tumors were not.[25,26] However, the activated drugs' range would ideally be limited enough to restrict it from traveling into and damaging normal tissues. In other words, the active drug should have local bystander effect, but very limited or no distal bystander effect.[27]

To try to ensure tumor cell–specific expression of the prodrug-activating enzymes, investigators have employed a number of methods including 1) intratumoral delivery, 2) tissue- or tumor-specific promoters (e.g., PSA, probasin), and 3) engineering of the relevant transgenes into replication-competent, tumor-selective viral systems under the control of the HCMV promoter[28] or under the control of a native viral promoter.[29] Whereas prodrug-based therapies have been administered using a variety of vectors into various cancers, these therapies have not generated meaningful benefit in the clinical setting, presumably due to the poor distribution of the replication-defective viruses used as delivery vehicles.[30] If this is the limitation to these therapies, replication-competent oncolytic viruses encoding prodrug-activating enzymes may prove to be highly effective as they have been shown to increase levels and distribution of genomically encoded factors over replication-defective viruses.[31]

Thymidine kinase (TK) and cytosine deaminase (CD) and their respective prodrugs [ganciclovir (GCV) and 5-flourocytosine (5-FC), respectively] are the most advanced of the prodrug-based therapies. Most recently, a gene fusion of CD/TK was engineered into a replication-competent, tumor-selective adenovirus and tested in a Phase I clinical trial on locally injectable prostatic tumors. The CD/TK fusion enzyme is a promising improvement for the oncolytic adenovirus because it saves genomic space, which is limited in adenovirus, without losing function.[32] In a 14-patient prostate cancer study, the virus was administered to the patients and, 2 days postinjection, the patients were given GCV and 5-FC, with the GCV and 5-FC dosing continuing for a total of 7 days. Two of 14 patients experienced full tumor regression, and an additional four patients had partial regressions (25–80% reduction in PSA levels). No dose-limiting toxicities were observed, and an MTD could not be reached.[28] These early trials indicate that this treatment, once optimized, may be both effective and safe.

Despite the encouraging initial data, prodrugs whose activated form interferes with DNA replication have been shown to limit the ability of the virus to continue to replicate and spread in the tumor.[33,34] These reductions in viral burst size mitigate the cytolytic potential of these viruses and potentially compromise the full utility of this approach. To avoid interference between the therapeutic effects of direct viral lysis and drug-induced cytotoxicity, other prodrugs less toxic to the virus or more optimized dosing schedules will need to be developed. If this can be achieved, these virotherapies should be able to build upon the already encouraging clinical data being generated around combination therapies with the virus and chemotherapy.[5] As this approach should result in reduced systemic toxicities normally associated with chemotherapy, this treatment may also be combined with other treatment modalities such as radiotherapy or immunotherapy. Much current evidence indicates that combined modalities are considerably more successful in fighting cancer than any of the component monotherapies.[5,26,32,34–40]

Antiangiogenic therapies

Unchecked cell proliferation is a hallmark of human cancers. The continued growth of the tumor, however, is dependent upon an adequate supply of oxygen and nutrients from the blood.[41,42] When tumor growth exceeds the normal blood supply to a tissue or organ, new blood vessel formation must be stimulated from surrounding existing vessels to support continued tumor growth. This process, termed tumor neovascularization (a special form of angiogenesis), consists of multiple steps and includes local degradation of the capillary basement membrane, recruitment and proliferation of endothelial cells, and remodeling and formation of a network of new blood vessels.

Tumor neovascularization is an appealing target for cancer therapeutics for several reasons. First, because neovascularization or angiogenesis is a necessity for tumor growth, antiangiogenics could be applied to any solid tumor, regard-

less of origin and independent of whether it is primary or metastatic disease. Second, because many of the angiogenesis inhibitors are "natural" (endogenous, nonsynthetic), these may be well tolerated by the patient in contrast to traditional chemotherapeutics or small molecules, for example.[43,44] Third, the target proliferating tumor endothelium differs significantly from the normal vascular endothelium in the adult. These differences range from proliferation rates (the normal vascular endothelium is quiescent in the adult, with turnover times measured in hundreds of days[45]) to gene expression profiles.[46] These differences offer potentially valuable targets for therapeutic intervention (see below). Lastly, resistance to angiogenesis inhibitors is less likely to occur. Genetic instability is one of the trademarks of the cancerous cell and is the mechanism responsible for acquisition of drug resistance in cancer cells. In contrast to the cancer cell, the target of angiogenic therapy is a normal, genetically stable endothelial cell stimulated to proliferate and migrate in response to angiogenic stimulus from the tumor. With its genetic stability still intact, the normal endothelial cell is less likely to acquire a mutation conveying therapeutic resistance. Consequently, the development of angiogenesis inhibitors, or inhibitors of tumor neovascularization, has become a broad and active area of cancer research (for recent reviews, see Refs. [47–49]).

More than 40 "natural" (endogenous, nonsynthetic) inhibitors of angiogenesis have been discovered and characterized.[48] The development of these inhibitors as therapeutic agents, however, has been hampered by several factors including manufacturing difficulties, and stability and solubility issues. In addition, the majority of these agents are not directly cytotoxic to tumor cells and so it is likely that these angiogenesis inhibitors would need to be expressed on a continuous basis. Gene therapy offers one potential avenue to address many of these issues. The finding that susceptibility to angiogenesis inhibitors can vary by tumor stage[50] and the recent disappointments of antiangiogenic matrix metalloproteinase inhibitors in the clinic[51] have caused investigators to begin to turn their attention to systems where angiogenesis inhibitors can be combined with standard or experimental cancer therapies.[52,53] In addition, more aggressive antiangiogenic therapies have begun to evolve in which investigators are developing systems to proactively eradicate the neovasculature[54,55] in contrast to arresting its growth. Consequently, it is timely to consider inhibitors of angiogenesis in the context of armed therapeutic viruses (oncolytic viruses encoding therapeutic transgenes). To date, however, replicating viruses encoding antiangiogenic therapeutic genes have not been reported.

Immunotherapy

The immune system is a complex mixture of effector molecules and cells that interact with one another to monitor and maintain the health of the host. Harnessing and targeting this potential into an effective therapy that selectively recognizes and eradicates the cancerous tissue remains a highly sought after, yet elusive, goal. Immunotherapy is based on the concept that there are differences between tumor cells and normal cells that can be detected by the immune system and can serve as targets for immune-mediated eradication of malignant disease. This is a very large and active field of gene therapy research and is at the center of the vast majority of the cancer gene therapy trials currently in the clinic. The use of cytokines, costimulatory molecules, and allogeneic major histocompatibility complex (MHC) molecules; the delivery of tumor antigens to dendritic cells (DCs); and the use of recombinant viruses expressing cancer antigens, alone or in combination with any of the previously described factors, all fall under this broad therapeutic umbrella directed at enhancing immune recognition, killing, and clearance of the target tumor cell.

These various strategies are commonly dependent on antigen-presenting cells (APCs) and cytotoxic T lymphocytes (CTLs). The APC is the sentinel for anomalies in the host. APCs include DCs, mononuclear–phagocytic cells, and activated B lymphocytes, with the DC serving as the target cell of choice for many cancer-based immunotherapies. This is because DCs are the most potent of the APCs, having a high capacity for antigen uptake in their immature form and high levels of MHC class I and II molecules, costimulatory molecules (B7-family), and adhesion molecules (ICAM-1, LFA-3, CD11a,c) in their mature form. These characteristics make them highly efficient at sampling the host environment, presenting antigen, and activating naïve T cells.[56–61] In addition, methods for collecting and growing DCs from hematopoietic precursors have been described[58,62,63] and serve to increase their attractiveness as contributors in a therapeutic strategy.

A robust antitumor CTL response has traditionally been the goal of the immunotherapy approach to cancer treatment. The value of the CTL stems from several factors. First, it is specific. Short peptides, 8–11 amino acids in length, derived from proteasome-digested intracellular proteins are shuttled into the endoplasmic reticulum (ER) by specialized transporters associated with antigen processing (TAP1 and TAP2) where they complex with MHC class I molecules. The MHC class I–peptide complex is consequently transported to the cell surface where it is recognized by the T-cell receptor (TCR) of the CTL. In an oversimplification of a complex process, if the APC has appropriately directed the maturation of a CTL that specifically recognizes a tumor antigen, the CTL will act to destroy the cell by one of two pathways. In the first, the CTL, upon antigen recognition, releases perforin and granzyme B, the perforin acting to create pores in the target cell membrane, which the granzyme penetrates to trigger a caspase-mediated apoptotic cascade.[64,65] An alternative pathway for CTL-mediated target cell killing involves a direct interaction between Fas ligand on the surface of the T lymphocyte and Fas receptor on the target cell, which also leads to caspase activation and apoptotic death of the target cell.[66,67] The cell killing event, then, is independent of other cell types and is, theoretically, long-lived, reducing the chance for reoccurrence of the disease.

How tumors evade recognition and clearance by these potent immune mechanisms remains controversial. Detection of tumor antigen–reactive $CD4^+$ and $CD8^+$ T cells and antibodies directed against a wide variety of tumor-associated gene products in human patients who nonetheless

have measurable cancer adds to the evidence that, like many checkpoints to neoplastic disease, the immune response can be circumvented by the human tumor.[68] Consequently it is important to consider several points when immunostimulatory factors and the immune system are considered in association with the replicating viral agent. First, tumor cells evade, manipulate, and proactively attack immune components in order to survive and proliferate. Evasion of the APC can take several forms. These range from tumor-associated factors that inhibit the differentiation, maturation, and/or function of DCs, e.g., VEGF, IL-6, M-CSF, IL-10, PGE_2, and TGF-β.[69,79] Decreased recognition (e.g., loss of MHC class I molecules, loss of peptide transporters, alterations in proteasome function), function (e.g., decreased levels of TCR signaling pathway proteins CD3ζ, $p56^{lck}$, $p59^{fyn}$, and impaired NF-κB activation), lack of appropriate stimuli (tolerance, clonal deletion), or T-cell survival (e.g., Fas ligand, MUC-1, B7-H1) have all been described as tumor-based mechanisms to evade CTL-mediated eradication.[71–85]

These immune-evasive strategies are daunting, but viral infection may be a key to breaking immune tolerance of tumors. It has been proposed that cancer cells are not detected, or quickly become immunologically tolerated, because they are generally not presented to the immune system in a microenvironment that favors the activation of immune cells. An oncolytic virus, then, is an interesting system to consider as a vehicle to generate a systemic immune response to the target tumor. This is, in part, because it is clear that viruses are highly immunogenic, as measured by high levels of antibody and T cells responses described in the normal population for many of the viruses being considered for development of oncolytic viruses. This suggests that the viral infection has the ability to supply "danger" signals, thought necessary to attract and initiate the DC-mediated process of antigen uptake and presentation that ultimately, in theory, leads to the generation of the tumor-specific CTL response. This is the basis for the use of poxvirus-based vaccines for cancer therapy[86] that are now in various stages of clinical trials. Several oncolytic viruses of Ad and HSV origin are being engineered to encode immunostimulatory cytokines in an attempt to enhance their potential at eliciting a systemic immune response that complements the lytic function of the virus.[87–91]

Oncolytic viruses may also break immune tolerance of tumors by reducing tumor burden (through viral lysis) to a point below which an anti-tumor immune response can be effective. Several studies have indicated that immune dysfunction can be correlated with total tumor burden.[32,81] An additional study has shown that the functional nature of the patient's immune response improved after debulking surgery.[92] Taken together, these studies indicate that lowering tumor burden through virus-induced cell death while stimulating antitumor immune response will increase the probability that a therapeutic systemic immune response will be elicited. Generating such a systemic immune response would be important to destroy metastatic disease.

While theoretically very inviting and well supported by preclinical studies, the ability to harness the immune system to generate long-term therapeutic benefit to the patient has not been realized yet in the clinic. Objective responses have been minimal and clear clinical benefit remains questionable. It should be noted that unlike classical vaccine studies performed prophylactically on healthy subjects, gene therapy–based cancer vaccine trials are faced with the challenge of generating an effective immune response to the target human tumor that has, by the time of its detection and the initiation of treatment, evolved in a variety of strategies to evade immune detection and eradication. It should also be noted that Phase I trials are conducted to determine the toxicity of the agent and are generally performed in late-stage patients who have failed chemotherapy, radiation therapy, and/or surgery. This may not be an ideal population for many of the therapies that require a robust immune response. It is hoped that the safety of these agents might justify offering this treatment to early-stage patients, who are expected to have a better chance of mounting a strong immune-based defense and thereby benefiting from these therapies.

Controlling therapeutic transgene expression from "armed" replicating oncolytic viruses

While it is important to consider the therapeutic factors and how they may synergize with the oncolytic virus to maximize therapeutic benefit, it is equally important to consider how these factors will be genetically engineered into their respective viral genomes and how their expression will be controlled. While packaging of therapeutic genes is generally not an issue for large viruses like HSV (nearly 50% of HSV genes are nonessential for viral replication[93]) and vaccinia (where it is estimated that the virus may be able to package approximately 50 kb of foreign DNA[86]), for smaller viruses like Ad, this is a considerable hurdle. For these viruses, gene delivery must be genomically economical. That is, consideration must be given to delivering as many therapeutic genes as possible from a genome that will only stably accommodate, in the case of Ad, approximately 2 kb of additional DNA beyond the size of the normal genome.[94] One strategy has been to generate multiple genes from a single transcript through the use of internal ribosome entry sites (IRESs),[95,96] which have been successfully employed in replicating viruses.[24,33] A second strategy offered by the replicating virus is to use the endogenous viral gene expression control machinery (promoter/enhancer, polyadenylation, and splice signals) to deliver transgenes and, where possible, to selectively replace an individual viral gene or genes with a therapeutic gene of choice. In this strategy, therapeutic transgene expression should follow the normal kinetics of the endogenous substituted gene. If the expression kinetics of the individual sites is diverse, this should enable investigators to tailor their therapeutic gene expression to levels and times they deem optimal to generate maximal therapeutic benefit. If these substitutions do not alter the remaining surrounding genes in a complex transcription unit and these genes are nonessential to the viral life cycle in the infected tumor cell, the investigator may be able to substitute the remaining genes with additional therapeutic genes. In this fashion, a combination of genes that target totally different aspects of tumor biology (e.g., prodrug-converting enzyme, immunostimulatory) could be incorpo-

rated into a single virus, synergizing with the inherent lytic property of the virus to attack the complexity of the tumor. This type of system has recently been described in the replicating Ad,[29,91,97,98] developed in the nonessential, immunoregulatory E3 region transcription unit.

Native viral promoters offer several advantages as the transgene expression system in the armed therapeutic virus. For example, many of the mechanisms to derive tumor specificity are genetically engineered to be the earliest events (i.e., attachment, penetration, immediate early gene expression) in the viral life cycle or are native to the virus. As the tumor-selective mechanism will dictate whether the viral life cycle is allowed to proceed, viral promoters whose expression follows that gating event will not be expressed in a normal, nontumor cell. Linking therapeutic gene expression to the selectivity of the virus should restrict therapeutic gene expression to the target tumor and should exclude it from occurring in nontarget tissue. This is a very important consideration for a systemically administered oncolytic virus targeted at metastatic disease, where a wide array of cells may be exposed to the agent. Thus, a strategy using endogenous late (in the viral life cycle) promoters offers a level of controlled expression in the oncolytic virus that would not be present if a constitutively active promoter (e.g., HCMV) were used.

Native viral promoters may also offer well-characterized gene expression kinetics[29,91,97] and native viral promoters are optimized for expression in the virally infected cell. With the correct choice of gene insertion sites, it has been shown that a replication-competent virus using a native unmodified viral promoter can achieve levels of therapeutic gene expression superior to those seen with the very strong HCMV promoter/enhancer generated from a replication-incompetent agent.[91]

Tissue- or tumor-specific promoters are also possibilities to convey tumor-specific therapeutic gene expression to the oncolytic virus. However, it is important to note that viral attachment and penetration events have the potential to make the nontarget normal cell appear to be a cancer cell to the tissue- or tumor-specific promoter. For example, the Ad penton protein (essential for penetration of the virus following attachment) interacts with $\alpha(v)$ integrins, and triggers PI 3 kinase activity.[93] The PI 3 kinases are considered an excellent target for cancer-based therapies because they initiate complex signaling cascades that mediate proliferation, differentiation, chemotaxis, and survival.[94,97,98] As this pathway is associated with cancer, it may affect a promoter's ability to discern whether the infected cell is "normal" or "tumor" in origin. This does not exclude using tissue- or tumor-specific promoters but will require careful examination of each promoter in the context of each individual virus for its specificity.

Challenges for armed, replicating, oncolytic virus-based therapies

The mechanisms of each of the various classes of gene-based therapeutics when used as monotherapies may be clear, but their potential interactions within the context of a replicating virus are not easily discerned. These interactions will either synergize to increase, or conflict to decrease, patient benefit. The actions of some therapeutic transgenes may synergize with one viral therapy, while interfering with another. Each combination therapy must be individually evaluated. For example, many of the gene-based therapeutic agents outlined previously in this review also have potential antiviral activities associated with them. In the case of the immunostimulatory factors, it is not only a consideration of the factor and its effect on the viral infection. There is also potential for redundant expression because the normal viral infection itself may stimulate various immunostimulatory factors (e.g., cytokines and chemokines). In this context, even if there is redundancy, the investigator will need to give careful consideration to the levels and duration of this effect before simply dismissing some of these seemingly overlapping, or redundant, factors. As most of the prodrug-converting enzymes are targeted towards DNA integrity and replication, these factors and their incorporation into the viral genome would appear to be a significant challenge, requiring careful consideration of the dosing regimen or control of expression of these factors. In the case of antiangiogenic factors, consideration should be given to whether viral replication will be affected by growth in hypoxic cells. This is not to suggest that these challenges cannot be overcome. Instead, these examples are meant to facilitate thought and discussion on how to overcome these potential hurdles as these therapies make their way towards the clinic, and to point to the fact that each therapeutic will require considerable thought to maximize its potency in the tumor microenvironment in association with the replicating virus.

Conclusion

Human tumors are complex entities that continue to challenge modern medicine to develop more effective cancer therapies. Replication-competent oncolytic viruses, either naturally occurring or genetically engineered, represent a new class of agents being developed and tested in the clinical[3,8,10–12,101,102] and preclinical settings.[103–105] These agents, with their capacity to amplify their dose through replication at the target site, then spread within the tumor to lyse neoplastic cells and decrease the tumor burden, represent unique anticancer therapeutics. It is not clear from past studies or from our current understanding of various potential viral agents which virus (or viruses) will best fulfill the oncolytic goals of sustained replication, exquisite specificity, and robust lytic activity when administered to the human tumor. Consequently, new oncolytic agents based on virus types already in the clinic (e.g., Ad, HSV, Newcastle disease virus, reovirus) or through alternative viruses (e.g., measles, poliovirus, VSV, vaccinia) must be explored. To effectively deal with the complex, heterogeneous nature of the tumor pool, however, the therapeutic transgene expression capacity of these viruses will likely also need to be developed. Armed therapeutic viruses, in which a therapeutic gene(s) is genetically engineered into the virus and dependent upon the continued selective replication of the

virus for expression, represent a very appealing tumor treatment and a novel opportunity to generate a single agent that can attack tumors at multiple levels. In addition, it allows the investigator the flexibility to engineer additional factors into the virus to overcome potential or identified deficiencies of the therapy in the clinical setting. It is important to note that treatment with an armed therapeutic virus does not exclude the use of chemotherapy, radiation, or surgery. To the contrary, as reviewed here, theoretical considerations and clinical trial data strongly support the use of these agents in combination with the viral-based therapy. Consequently, armed therapeutic viruses represent a potentially exciting new treatment paradigm for human cancers.

References

1. Wildner O. Oncolytic viruses as therapeutic agents. *Ann Med.* 2001;33:291–304.
2. Ring CJ. Cytolytic viruses as potential anti-cancer agents. *J Gen Virol.* 2002;83(Pt 3):491–502.
3. Kirn D. Replication-selective oncolytic adenoviruses: virotherapy aimed at genetic targets in cancer. *Oncogene.* 2000; 19:6660–6669.
4. Hermiston T. Gene delivery from replication-selective viruses: arming guided missiles in the war against cancer. *J Clin Invest.* 2000;105:1169–1172.
5. Khuri FR, Nemunaitis J, Ganly I, et al. A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med.* 2000;6:879–885.
6. Smith RR, Huebner RJ, Rowe WP, Schatten WE, Thomas LB. Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer.* 1956;9:1211–1218.
7. Kirn D. Replication-selective oncolytic adenoviruses: virotherapy aimed at genetic targets in cancer. *Oncogene.* 2000; 19:6660–6669.
8. Reid T, Galanis E, Abbruzzese J, et al. Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: a phase I trial. *Gene Ther.* 2001;8:1618–1626.
9. Nemunaitis J, Khuri F, Ganly I, et al. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol.* 2001;19:289–298.
10. Markert JM, Medlock MD, Rabkin SD, et al. Conditionally replicating herpes simplex virus mutant, G207, for the treatment of malignant glioma: results of a phase I trial. *Gene Ther.* 2000;7:867–874.
11. Rampling R, Cruickshank G, Papanastassiou V, et al. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.* 2000;7:859–866.
12. Pecora AL, Rizvi N, Cohen GI, et al. Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J Clin Oncol.* 2002;20: 2251–2266.
13. Csatary LK, Moss RW, Beuth J, et al. Beneficial treatment of patients with advanced cancer using a Newcastle disease virus vaccine (MTH-68/H). *Anticancer Res.* 1999;19:635–638.
14. Csatary LK, Bakacs T. Use of Newcastle disease virus vaccine (MTH-68/H) in a patient with high-grade glioblastoma. *JAMA.* 1999;281:1588–1589.
15. Kirn D, Niculesco-Duvaz I, Halden G, Springer CJ. The emerging fields of suicide gene therapy and virotherapy. *Trends Mol Med.* 2002;8:S68–S73.
16. Hermiston T. Fighting fire with fire: attacking the complexity of human tumors with armed therapeutic viruses. *Curr Opin Mol Ther.* 2002;4:334–342.
17. McCormick F. Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer.* 2001;1:130–141.
18. Wilson DR. Viral-mediated gene transfer for cancer treatment. *Curr Pharmacol Biotechnol.* 2002;3:151–164.
19. Willis AC, Chen X. The promise and obstacle of p53 as a cancer therapeutic agent. *Curr Mol Med.* 2002;2:329–345.
20. Levine A, DiMiao D, Coen DM. *Fields Virology* (Fourth Edition). 2001;1:3–18, 119–132.
21. Nishizaki M, Fujiwara T, Tanida T, et al. Recombinant adenovirus expressing wild-type p53 is antiangiogenic: a proposed mechanism for bystander effect. *Clin Cancer Res.* 1999;5:1015–1023.
22. Buckbinder L, Talbott R, Velasco-Miguel S, et al. Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature.* 1995;377:646–649.
23. Mueller H. Tumor necrosis factor as an antineoplastic agent: pitfalls and promises. *Cell Mol Life Sci.* 1998;54:1291–1298.
24. Sauthoff H, Pipiya T, Heitner S, et al. Late expression of p53 from a replicating adenovirus improves tumor cell killing and is more tumor cell specific than expression of the adenoviral death protein. *Hum Gene Ther.* 2002 (In press).
25. Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.* 1986;46:5276–5281.
26. Stribbling SM, Friedlos F, Martin J, et al. Regressions of established breast carcinoma xenografts by carboxypeptidase G2 suicide gene therapy and the prodrug CMDA are due to a bystander effect. *Hum Gene Ther.* 2000;11:285–292.
27. Knox RJ. Gene-directed enzyme prodrug therapy (GDEPT) — recognizing the present limitations of gene therapy for the treatment of cancer. *Curr Opin Invest Drugs.* 2001;2:835–838.
28. Freytag SO, Kim JH, Khil MS, et al. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for local recurrence of prostate cancer. *Proc Am Assoc Cancer Res.* 2002;43 (Abstract 5429).
29. Hawkins LK, Hermiston T. Gene delivery from the E3 region of replicating human adenovirus: evaluation of the E3B region. *Gene Ther.* 2001;8:1142–1148.
30. van Dillen IJ, Mulder NH, Vaalburg W, de Vries EF, Hospers GA. Influence of the bystander effect on HSV-tk/GCV gene therapy. A review. *Curr Gene Ther.* 2002;2: 307–322.
31. Ichikawa T, Chiocca EA. Comparative analyses of transgene delivery and expression in tumors inoculated with a replication-conditional or defective viral vector. *Cancer Res.* 2001;61:5336–5339.
32. Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther.* 1998;9:1323–1333.
33. Wildner O, Morris JC. Therapy of peritoneal carcinomatosis from colon cancer with oncolytic adenoviruses. *J Gene Med.* 2000;2:353–360.
34. McCart JA, Puhlmann M, Lee J, et al. Complex interactions between the replicating oncolytic effect and the enzyme/prodrug effect of vaccinia-mediated tumor regression. *Gene Ther.* 2000;7: 1217–1223.
35. Ichikawa T, Petros WP, Ludeman SM, et al. Intraneoplastic

polymer-based delivery of cyclophosphamide for intratumoral bioconversion by a replicating oncolytic viral vector. *Cancer Res.* 2001;61: 864–868.
36. Lee YJ, Galoforo SS, Battle P, et al. Replicating adenoviral vector-mediated transfer of a heat-inducible double suicide gene for gene therapy. *Cancer Gene Ther.* 2001;8:397–404.
37. Nemunaitis J, Swisher SG, Timmons T, et al. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol.* 2000;18:609–622.
38. Sanchez-Prieto R, Quintanilla M, Cano A, et al. Carcinoma cell lines become sensitive to DNA-damaging agents by the expression of the adenovirus E1A gene. *Oncogene.* 1996;13:1083–1092.
39. Toyoizumi T, Mick R, Abbas AE, et al. Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. *Hum Gene Ther.* 1999;10:3013–3029.
40. You L, Yang CT, Jablons DM. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res* 2000;60:1009–1013.
41. Folkman J. Tumor angiogenesis. *Adv Cancer Res.* 1985; 43:175–203.
42. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst.* 1990;82:4–6.
43. Thompson WD, Li WW, Maragoudakis M. The clinical manipulation of angiogenesis: pathology, side-effects, surprises, and opportunities with novel human therapies. *J Pathol.* 2000;190:330–337.
44. Folkman J. Therapeutic angiogenesis in ischemic limbs. *Circulation.* 1998;97:1108–1110.
45. Denekamp J. Vascular attack as a therapeutic strategy for cancer. *Cancer Metastasis Rev.* 1990;9:267–282.
46. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science.* 2000;289:1197–1202.
47. Talks KL, Harris AL. Current status of antiangiogenic factors. *Br J Haematol.* 2000;109:477–489.
48. Feldman AL, Libutti SK. Progress in antiangiogenic gene therapy of cancer. *Cancer.* 2000;89:1181–1194.
49. Kleinman HK, Liau G. Gene therapy for antiangiogenesis. *J Natl Cancer Inst.* 2001;93:965–967.
50. Bergers G, Javaherian K, Lo KM, Folkman J, Hanahan D. Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science.* 1999;284:808–812.
51. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science.* 2002;295:2387–2392.
52. Lund EL, Bastholm L, Kristjansen PE. Therapeutic synergy of TNP-470 and ionizing radiation: effects on tumor growth, vessel morphology, and angiogenesis in human glioblastoma multiforme xenografts. *Clin Cancer Res.* 2000; 6:971–978.
53. Bello L, Carrabba G, Giussani C, et al. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth *in vivo*. *Cancer Res.* 2001;61:7501–7506.
54. Hood JD, Bednarski M, Frausto R, et al. Tumor regression by targeted gene delivery to the neovasculature. *Science.* 2002; 296:2404–2407.
55. Savontaus MJ, Sauter BV, Huang TG, Woo SL. Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells. *Gene Ther.* 2002;9:972–979.
56. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol.* 1991;9:271–296.
57. Steinman RM. Dendritic cells and immune-based therapies. *Exp Hematol.* 1996;24:859–862.
58. Steinman RM, Pack M, Inaba K. Dendritic cell development and maturation. *Adv Exp Med Biol.* 1997;417:1–6.
59. Lanzavecchia A. Identifying strategies for immune intervention. *Science.* 1993;260:937–944.
60. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998;392:245–252.
61. Dallal RM, Lotze MT. Immunotherapy of metastasis. *Surg Oncol Clin North Am.* 2001;10:433–447, xi.
62. Caux C, Massacrier C, Vanbervliet B, et al. $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte–macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood.* 1997;90:1458–1470.
63. Young JW, Szabolcs P, Moore MA. Identification of dendritic cell colony-forming units among normal human $CD34^+$ one marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J Exp Med.* 1995;182:1111–1119.
64. Chinnaiyan AM, Hanna WL, Orth K, et al. Cytotoxic T-cell–derived granzyme B activates the apoptotic protease ICE-LAP3. *Curr Biol.* 1996; 6:897–899.
65. Froelich CJ, Dixit VM, Yang X. Lymphocyte granule–mediated apoptosis: matters of viral mimicry and deadly proteases. *Immunol Today.* 1998;19:30–36.
66. Alderson MR, Tough TW, Davis-Smith T, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med.* 1995;181: 71–77.
67. Lynch DH, Alderson MR, Ramsdell F. Immunoregulatory effects of Fas-mediated signalling. *J Cell Biochem.* 1996;60: 39–46.
68. Dranoff G. Tumor immunology: immune recognition and tumor protection. *Curr Opin Immunol.* 2002;14:161–164.
69. Vicari AP, Caux C. Chemokines in cancer. *Cytokine Growth Factor Rev.* 2002;13:143–154.
70. Vicari AP, Caux C, Trinchieri G. Tumour escape from immune surveillance through dendritic cell inactivation. *Semin Cancer Biol.* 2002;12:33–42.
71. Hellstrom I, Hellstrom KE. Tumor immunology: an overview. *Ann NY Acad Sci.* 1993;690:24–33.
72. Hellstrom KE, Hellstrom I, Linsley P, Chen L. On the role of costimulation in tumor immunity. *Ann NY Acad Sci.* 1993;690:225–230.
73. Nakagomi H, Petersson M, Magnusson I, et al. Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res.* 1993; 53:5610–5612.
74. Finke JH, Zea AH, Stanley J, et al. Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma. *Cancer Res.* 1993;53:5613–5616.
75. Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today.* 1995;16:487–494.
76. Seliger B, Maeurer MJ, Ferrone S. TAP off-tumors on. *Immunol Today.* 1997;18:292–299.
77. Hersey P. Impediments to successful immunotherapy. *Pharmacol Ther.* 1999;81:111–119.
78. Morford LA, Elliott LH, Carlson SL, Brooks WH, Roszman TL. T cell receptor–mediated signaling is defective in T cells obtained from patients with primary intracranial tumors. *J Immunol.* 1997;159:4415–4425.
79. Sogn JA. Tumor immunology: the glass is half full. *Immunity.* 1998;9:757–763.

80. Ling W, Rayman P, Uzzo R, et al. Impaired activation of NFkappaB in T cells from a subset of renal cell carcinoma patients is mediated by inhibition of phosphorylation and degradation of the inhibitor, IkappaBalpha. *Blood*. 1998;92: 1334–1341.
81. Drake CG, Pardoll DM. Tumor immunology — towards a paradigm of reciprocal research. *Semin Cancer Biol*. 2002; 12:73–80.
82. Pardoll DM. Spinning molecular immunology into successful immunotherapy. *Nat Rev Immunol*. 2002;2:227–238.
83. O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. Resistance to Fas (APO-1/CD95)–mediated apoptosis and expression of Fas ligand in esophageal cancer: the Fas counterattack. *Dis Esophagus*. 1999; 12:83–89.
84. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793–800.
85. Gimmi CD, Morrison BW, Mainprice BA, et al. Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. *Nat Med*. 1996;2:1367–1370.
86. Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc Natl Acad Sci USA*. 1996;93:11341–11348.
87. Andreansky S, He B, van Cott J, et al. Treatment of intracranial gliomas in immunocompetent mice using herpes simplex viruses that express murine interleukins. *Gene Ther*. 1998;5:121–130.
88. Kurihara T, Brough DE, Kovesdi I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J Clin Invest*. 2000; 106:763–771.
89. Wong RJ, Patel SG, Kim S, et al. Cytokine gene transfer enhances herpes oncolytic therapy in murine squamous cell carcinoma. *Hum Gene Ther*. 2001;12:253–265.
90. Bennett JJ, Malhotra S, Wong RJ, et al. Interleukin 12 secretion enhances antitumor efficacy of oncolytic herpes simplex viral therapy for colorectal cancer. *Ann Surg*. 2001;233:819–826.
91. Hawkins LK, Johnson L, Bauzon M, et al. Gene delivery from the E3 region of replicating human adenovirus: evaluation of the 6.7 K/gp19 K region. *Gene Ther*. 2001;8:1123–1131.
92. Heriot AG, Marriott JB, Cookson S, Kumar D, Dalgleish AG. Reduction in cytokine production in colorectal cancer patients: association with stage and reversal by resection. *Br J Cancer*. 2000;82:1009–1012.
93. Roizman B. The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors. *Proc Natl Acad Sci USA*. 1996;93:11307–11312.
94. Kiessling R, Wasserman S, Horiguchi S, et al. Tumor-induced immune dysfunction. *Cancer Immunol Immunother*. 1999;48:353–362.
95. Li E, Stupack D, Bokuch GM, Nemerow GR. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol*. 1998;72:8806–8812.
96. Roymans D, Slegers H. Phosphatidylinositol 3-kinases in tumor progression. *Eur J Biochem*. 2001;268:487–498.
97. Hawkins LK, Hermiston TW. Gene delivery from the E3 region of replicating human adenovirus: evaluation of the ADP region. *Gene Ther*. 2001;8:1132–1141.
98. Nanda D, Vogels R, Havenga M, et al. Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. *Cancer Res*. 2001;61:8743–8750.
99. Katso R, Okkenhaug K, Ahmadi K, et al. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*. 2001;17:615–675.
100. Berrie CP. Phosphoinositide 3-kinase inhibition in cancer treatment. *Expert Opin Invest Drugs*. 2001;10:1085–1098.
101. Hawkins LK, Lemoine NR, Kirn D. Oncolytic biotherapy: a novel therapeutic platform. *Lancet Oncol*. 2002;3:17–26.
102. Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat Med*. 2001;7:781–787.
103. Timiryasova TM, Li J, Chen B, et al. Antitumor effect of vaccinia virus in glioma model. *Oncol Res*. 1999;11:133–144.
104. Coffey MC, Strong JE, Forsyth PA, Lee PW. Reovirus therapy of tumors with activated Ras pathway. *Science*. 1998;282:1332–1334.
105. Stojdl DF, Lichty B, Knowles S, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med*. 2000;6:821–825.
106. Ganly I, Kirn D, Eckhardt G, et al. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res*. 2000; 6:798–806.
107. Vasey PA, Shulman LN, Campos S, et al. Phase I trial of intraperitoneal injection of the E1B–55-kd-gene–deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol*. 2002;20:1562–1569.
108. Bischoff JR, Kirn DH, Williams A, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science*. 1996;274:373–376.
109. Mulvihill S, Warren R, Venook A, et al. Safety and feasibility of injection with an E1B–55 kDa gene–deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther*. 2001;8:308–315.
110. Habib N, Salama H, Abd El Latif Abu Median A, et al. Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. *Cancer Gene Ther*. 2002;9:254–259.
111. Freytag SO, Khil M, Stricker H, et al. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res*. 2002;62:4968–4976.
112. Wildner O, Blaese RM, Morris JC. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. *Cancer Res*. 1999;59: 410–413.
113. Heise C, Hermiston T, Johnson L, et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med*. 2000;6:1134–1139.
114. Fueyo J, Gomez-Manzano C, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. *Oncogene*. 2000;19:2–12.
115. Suzuki K, Fueyo J, Krasnykh V, et al. A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clin Cancer Res*. 2001;7:120–126.
116. Howe JA, Demers GW, Johnson DE, et al. Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. *Mol Ther*. 2000;2:485–495.
117. Doronin K, Toth K, Kuppuswamy M, et al. Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J Virol*. 2000;74:6147–6155.
118. Doronin K, Kuppuswamy M, Toth K, et al. Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J Virol*. 2001;75:3314–3324.

119. DeWeese TL, van der Poel H, Li S, et al. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* 2001;61: 7464–7472.
120. Chen Y, DeWeese T, Dilley J, et al. CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. *Cancer Res.* 2001;61:5453–5460.
121. Yu DC, Chen Y, Dilley J, et al. Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res.* 2001;61:517–525.
122. Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. *Gene Ther.* 2002;9:270–281.
123. Johnson L, Shen A, Boyle L, et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell.* 2002;1:325–337.
124. Hallenbeck PL, Chang YN, Hay C, et al. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther.* 1999; 10:1721–1733.
125. Nettelbeck DM, Rivera AA, Balague C, Alemany R, Curiel DT. Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter. *Cancer Res.* 2002;62:4663–4670.
126. Ramachandra M, Rahman A, Zou A, et al. Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy. *Nat Biotechnol.* 2001;19:1035–1041.
127. Tsukuda K, Wiewrodt R, Molnar-Kimber K, Jovanovic VP, Amin KM. An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: potent antitumoral efficacy but no toxicity to normal cell. *Cancer Res.* 2002;62:3438–3447.
128. Takahashi M, Sato T, Sagawa T, et al. E1B–55K–deleted adenovirus expressing E1A-13S by AFP-enhancer/promoter is capable of highly specific replication in AFP-producing hepatocellular carcinoma and eradication of established tumor. *Mol Ther.* 2002;5:627–634 (Part 1).
129. Fabra A, Parada C, Vinyals A, et al. Intravascular injections of a conditional replicative adenovirus (adl118) prevent metastatic disease in human breast carcinoma xenografts. *Gene Ther.* 2001;8: 1627–1634.
130. Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med.* 1995;1:938–943.
131. MacLean AR, ul-Fareed M, Robertson L, Harland J, Brown SM. Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17' between immediate early gene 1 and the "a" sequence. *J Gen Virol.* 1991;72:631–639.
132. Delman KA, Bennett JJ, Zager JS, et al. Effects of preexisting immunity on the response to herpes simplex–based oncolytic viral therapy. *Hum Gene Ther.* 2000;11:2465–2472.
133. Pyles RB, Warnick RE, Chalk CL, Szanti BE, Parysek LM. A novel multiply-mutated HSV-1 strain for the treatment of human brain tumors. *Hum Gene Ther.* 1997; 8:533–544.
134. Parker LP, Wolf JK, Price JE. Adenoviral-mediated gene therapy with Ad5CMVp53 and Ad5CMVp21 in combination with standard therapies in human breast cancer cell lines. *Ann Clin Lab Sci.* 2000;30:395–405.
135. Fu X, Zhang X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. *Cancer Res.* 2002;62:2306–2312.
136. Chase M, Chung RY, Chiocca EA. An oncolytic viral mutant that delivers the CYP2B1 transgene and augments cyclophosphamide chemotherapy. *Nat Biotechnol.* 1998;16: 444–448.
137. Pawlik TM, Nakamura H, Mullen JT, et al. Prodrug bioactivation and oncolysis of diffuse liver metastases by a herpes simplex virus 1 mutant that expresses the CYP2B1 transgene. *Cancer.* 2002;95: 1171–1181.
138. Mineta T, Rabkin SD, Martuza RL. Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. *Cancer Res.* 1994;54:3963–3966.
139. Boviatsis EJ, Park JS, Sena-Esteves M, et al. Long-term survival of rats harboring brain neoplasms treated with ganciclovir and a herpes simplex virus vector that retains an intact thymidine kinase gene. *Cancer Res.* 1994;54:5745–5751.
140. Todo T, Martuza RL, Rabkin SD, Johnson PA. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. *Proc Natl Acad Sci USA.* 2001;98:6396–6401.
141. Miyatake S, Iyer A, Martuza RL, Rabkin SD. Transcriptional targeting of herpes simplex virus for cell-specific replication. *J Virol.* 1997;71:5124–5132.
142. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science.* 1991;252:854–856.
143. Chung RY, Saeki Y, Chiocca EA. B-myb promoter retargeting of herpes simplex virus gamma34.5 gene-mediated virulence toward tumor and cycling cells. *J Virol.* 1999;73: 7556–7564.
144. Nakamura H, Mullen JT, Chandrasekhar S, et al. Multimodality therapy with a replication-conditional herpes simplex virus 1 mutant that expresses yeast cytosine deaminase for intratumoral conversion of 5-fluorocytosine to 5-fluorouracil. *Cancer Res.* 2001;61: 5447–5452.
145. Markert JM, Malick A, Coen DM, Martuza RL. Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. *Neurosurgery.* 1993;32:597–603.
146. Whitman ED, Tsung K, Paxson J, Norton JA. *In vitro* and *in vivo* kinetics of recombinant vaccinia virus cancer-gene therapy. *Surgery.* 1994;116:183–188.
147. Puhlmann M, Gnant M, Brown CK, Alexander HR, Bartlett DL. Thymidine kinase–deleted vaccinia virus expressing purine nucleoside phosphorylase as a vector for tumor-directed gene therapy. *Hum Gene Ther.* 1999;10:649–657.
148. Peplinski GR, Tsung K, Meko JB, Norton JA. Prevention of murine breast cancer by vaccination with tumor cells modified by cytokine-producing recombinant vaccinia viruses. *Ann Surg Oncol.* 1996;3:15–23.
149. Peplinski GR, Tsung K, Casey MJ, et al. *In vivo* murine tumor gene delivery and expression by systemic recombinant vaccinia virus encoding interleukin-1beta. *Cancer J Sci Am.* 1996;2:21.
150. Mastrangelo MJ, Maguire HC Jr, Eisenlohr LC, et al. Intratumoral recombinant GM-CSF–encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther.* 1999;6:409–422.
151. Gnant MF, Puhlmann M, Alexander HR Jr, Bartlett DL. Systemic administration of a recombinant vaccinia virus

expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. *Cancer Res.* 1999;59:3396–3403.
152. Mukherjee S, Haenel T, Himbeck R, et al. Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. *Cancer Gene Ther.* 2000;7:663–670.
153. Hodge JW, Sabzevari H, Yafal AG, et al. A triad of costimulatory molecules synergize to amplify T-cell activation. *Cancer Res.* 1999;59:5800–5807.
154. McCart JA, Ward JM, Lee J, et al. Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res.* 2001;61:8751–8757.
155. Norman KL, Coffey MC, Hirasawa K, et al. Reovirus oncolysis of human breast cancer. *Hum Gene Ther.* 2002;13: 641–652.
156. Gromeier M, Lachmann S, Rosenfeld MR, Gutin PH, Wimmer E. Intergeneric poliovirus recombinants for the treatment of malignant glioma. *Proc Natl Acad Sci USA.* 2000;97:6803–6808.
157. Balachandran S, Porosnicu M, Barber GN. Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis. *J Virol.* 2001;75:3474–3749.
158. Peng KW, TenEcyck CJ, Galanis E, et al. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res.* 2002;62: 4656–4662.
159. Peng KW, Ahmann GJ, Pham L, et al. Systemic therapy of myeloma xenografts by an attenuated measles virus. *Blood.* 2001;98:2002–2007.